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TITLE: Phase I "Bioactive Lipids: Role in Prostate Cancer  
Angiogenesis"

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<b>13. ABSTRACT (Maximum 200 Words)</b>  The goal of DAMD17-98-1-8502 Idea Development Award is to investigate the role of 12-lipoxygenase (LOX) and its arachidonate product, 12(S)-hydroxyeicosatetraenoic acid (HETE), in human prostate cancer angiogenesis. The research progress during the funding period confirms the role of 12-LOX as a novel "angiogenic switch" governing prostate cancer angiogenesis and tumor growth. We found that 12-LOX increased the angiogenic potential of PCa cells by stimulating the production of VEGF, in addition to its arachidonate product 12(S)-HETE. The link between 12-LOX, a free fatty acid metabolizing enzyme, and VEGF, a putative angiogenic factor, is both novel and exciting, providing significant insights into our understanding of the regulation of VEGF expression during PCa progression. The study also found that 12-LOX and its lipid product, 12(S)-HETE, regulate VEGF expression at transcriptional level. The study also demonstrated the plausibility of using 12-LOX inhibitors such as BMD188 to inhibit prostate tumor angiogenesis and growth.				
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## INTRODUCTION

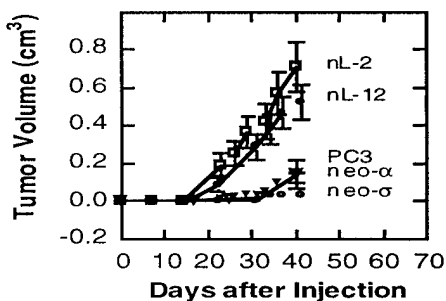
The goal of DAMD17-98-1-8502 Idea Development Award is to investigate the role of 12-lipoxygenase (LOX) and its arachidonate product, 12(S)-hydroxyeicosatetraenoic acid (HETE), in human prostate cancer angiogenesis. In the first 18-month funding period, we found that 12-LOX, when constitutively expressed in PC-3 cells, increased the ability of cancer cells to stimulate endothelial cell migration in vitro and angiogenesis in vivo and the ability to form larger, rapidly growing tumors in an animal model. Further it was found that 12-LOX functions as an "angiogenic switch" in PCa cells by stimulating the production of 12(S)-HETE and vascular endothelial growth factor (VEGF). 12(S)-HETE activates p42/44 MAP kinase in endothelial cells and stimulates endothelial cell migration. Further, 12(S)-HETE enhances the secretion of VEGF in PC-3 cells. Increased VEGF gene expression in 12-LOX transfected PC-3 cells was confirmed by ELISA measurement of VEGF levels, Northern Blot and VEGF promoter activity analysis. These observations, made in the first 18-month of funding, support our hypothesis that 12-LOX play a contributory role in prostate cancer angiogenesis. The findings also significantly further our understanding of prostate cancer angiogenesis in general and lipid regulation of angiogenesis in particular. The findings also identify 12-LOX as a novel target for developing anti-angiogenesis therapy for human prostate cancer.

## BODY

**Task 1.** Corroborate the preliminary data regarding increased vascularization of tumors formed by 12-LOX transfected PC3 cells vs. neocontrols in two additional PC3 transfectant clones and determine the vessel density, proliferation index, and apoptotic index in these tumors, Months 1-18:

This task has been achieved. Specifically, we have confirmed an increase in the growth of tumors derived from 12-LOX transfected PC-3 cells with three independent clones, nL-2, nL-8, and nL-12, when compared to neo- $\alpha$ , neo- $\sigma$ , or PC-3 parental cells. H&E staining revealed a decrease in necrosis and a slight increase in mitotic index in tumors derived from 12-LOX transfected PC-3 cells. The slight increase in mitotic index was further corroborated with MIB-1 immunostaining. TUNEL assay revealed a decrease in apoptosis in tumors derived from 12-LOX transfected PC-3 cells. CD31 immunostaining revealed an increase in vessel density and organization in tumors derived from 12-LOX transfected PC-3 cells, when compared with neo-control tumors (Cancer Res. 58: 4047, 1998).

During Phase I of this grant, we found that 12-LOX can serve as a novel “angiogenic switch” governing human prostate tumor growth and angiogenesis (Nie et al., 1998). As an enzyme, 12-LOX metabolizes arachidonic acid, a 20-C unsaturated free fatty acid, into 12(S)-HETE. Expression of this enzyme has been found in prostate cancer (Gao et al., 1995), pancreatic cancer (Ding et al., 1999), breast cancer (Natarajan et al., 1997; Connolly and Rose, 1998) and lung cancer (Chen et al., 1994), among others (Hong et al., 1999). During Phase I of this grant, we over-expressed 12-LOX in human PCa PC-3 cells by transfection with a platelet-type 12-LOX cDNA construct. Stable transfectants, which constitutively express high levels of 12-LOX at both the mRNA and protein levels, were generated and cloned. These 12-LOX transfected PC-3 cells produced more 12(S)-HETE than did the mock-transfected PC-3 cells. We found that, *in vitro*, the growth rates of several 12-LOX transfected PC-3 cells were similar to those of neo-controls and PC-3 wild type. However, following s.c. injection into nude mice, 12-LOX transfected PC-3 cells (nL2 and nL-12) grew faster and formed larger tumors than neo-controls (neo- $\alpha$  and neo- $\sigma$ ) (**Figure 1**) and the increased tumor volume was positively correlated with increased MVD as well as enhanced vessel organizations in tumor tissue (Nie et al., 1998).

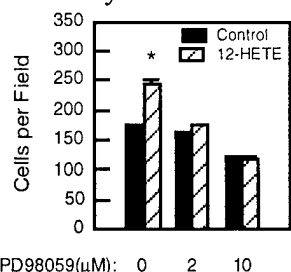


**Figure 1.** Growth kinetics of tumors derived from 12-LOX transfected PC-3 cells and their vector controls.

It should be noted that similar findings regarding the role of 12-LOX in tumor angiogenesis and growth were independently reported by Connolly and Rose (1998) in human breast cancer. The studies suggest that 12-LOX in cancer cells enhances their angiogenic potential. This novel function of 12-LOX, together with the observation that in human PCa the level of 12-LOX mRNA expression is correlated with tumor stage and grade (Gao et al., 1995), suggests that 12-LOX may serve as a key “angiogenic switch” to stimulate and sustain angiogenesis during human PCa progression.

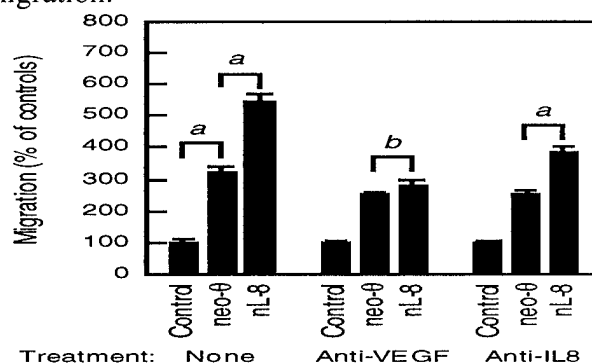
**Task 2.** Determine *in vitro* using endothelial cell migration assay that the observed increase in RV-ECT migration to PC3 12-LOX transfectants is due wholly or partly to increased 12-LOX activity, Months 1-12:

This task has been achieved. We found that 12(S)-HETE stimulates endothelial cell migration in a dose-dependent manner as reported in Cancer Res. 58: 4047 (1998). Further, we found that 12(S)-HETE activates p42/44 MAP kinase in endothelial cells and inhibition of the activation of p42/44 MAP kinase by PD98059 abrogated 12(S)-HETE stimulated endothelial cell migration (**Figure 2**). We also found that other isomers such as 5(S)-HETE, 15(S)-HETE, or 12(R)-HETE did not significantly stimulate endothelial cell migration, as reported in Blood 95: 2304 (2000). To study whether 12-LOX transfected PC3 cells can secrete protein factors, in addition to bioactive lipid 12(S)-HETE, to stimulate endothelial cell migration, the spent culture media were concentrated 8-fold using centricon-10 to eliminate small molecules and examined for the ability of the concentrates to stimulate endothelial cell migration.



**Figure 2.** Erk1/2 Dependent Stimulation of RV-ECT Cell Migration by 12(S)-HETE. \*,  $P < 0.05$  when compared to its control.

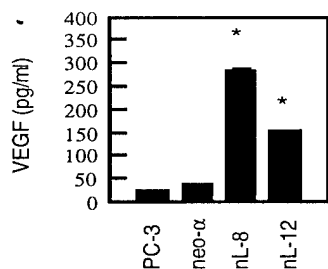
As shown in **Figure 3**, the culture media from 12-LOX transfected PC-3 cells still retained the ability to stimulate endothelial cell migration even after 12(S)-HETE was greatly depleted, suggesting the involvement of additional angiogenic factors, in addition to 12(S)-HETE, in the increased angiogenicity of 12-LOX transfected PC-3 cells.



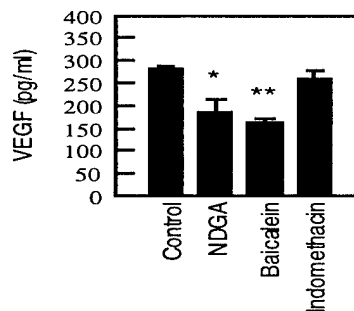
**Figure 3.** Stimulation of Endothelial Cell Migration by nL8 is Partially Mediated by VEGF. a,  $P < 0.01$ ; b,  $P > 0.05$ .

**Task 3.** *If the increased endothelial cell migration cannot be accounted for in total by increased 12(S)-HETE production, then we will determine if there is increased expression of putative angiogenic factors or decreased levels of natural angiogenesis inhibitors in 12-LOX transfectants, Months 12-24:*

This task has been achieved. ELISA analysis of the conditioned media revealed an increase in VEGF levels in 12-LOX transfected PC-3 cells (nL-8 and nL-12) (**Figure 4**). We also measured the levels of IL-8 and bFGF and found no change in IL-8 levels in 12-LOX transfected PC-3 cells, as compared to their neo-controls or PC-3 parental cells (data not shown). Neither did we detect any change in bFGF levels in culture media or cell lysates (data not shown). The data suggest that 12-LOX stimulate VEGF secretion, but not IL-8 or bFGF, from PC-3 cells. As shown in the **Figure 3**, in the absence of any neutralizing antibody, neo-θ PC-3 cells produced factors (MW > 10 kDa) to stimulate endothelial cell migration (please compare neo-θ with the control). 12-LOX transfected PC-3 cells (nL8) produced much more such factors than neo-θ to stimulate endothelial cell migration ( $P < 0.01$ ). This increased ability was reduced to insignificant level ( $P > 0.05$ ) by pretreating the concentrates with a VEGF neutralizing antibody. An IL-8 neutralizing antibody reduced this increased ability of nL-8 to some extent, but nL-8 still retained significant increased angiogenic ability in the presence of IL-8 neutralizing antibody (**Figure 3**).



**Figure 4.** Increased VEGF Expression in 12-LOX Transfected PC-3 Cells. \*,  $P < 0.01$ .

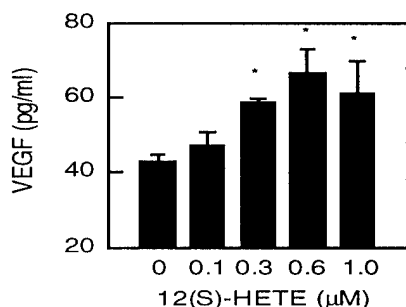


**Figure 5.** Down-regulation of VEGF Production in nL-8 by 12-LOX Inhibitors. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , when compared to the control.

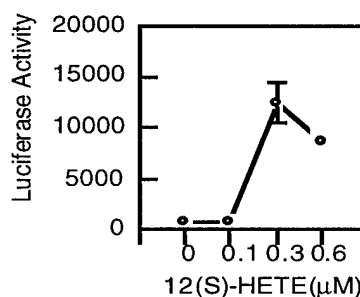
Treatment of 12-LOX transfected PC-3 cells with either NDGA (50  $\mu$ M), a general LOX inhibitors, or baicalein (10  $\mu$ M), a specific platelet-type 12-LOX inhibitor, significantly decreased VEGF expression while a general COX inhibitor, indomethacin (50  $\mu$ M), had no appreciable effect (**Figure 5**).

**Task 4.** Determine *in vitro* if exogenous 12(S)-HETE can enhance the expression of the angiogenic factor(s) or reduce the levels of endogenous inhibitor(s) identified in the previous Objective, Months 18-30:

This task has been met. We found that 12(S)-HETE increase VEGF expression in a dose-dependent manner (**Figure 6**). Further, 12(S)-HETE also stimulated VEGF promoter activity (**Figure 7**). However, it should be noted that the extent of stimulation of VEGF expression by 12(S)-HETE is far less than that observed in 12-LOX transfected PC-3 cells, suggesting that exogenously added 12(S)-HETE has mimicked to certain extent, but cannot duplicate the cellular functions of 12-LOX or endogenously produced 12(S)-HETE.



**Figure 6.** Stimulation of VEGF secretion in PC3 cells by 12(S)-HETE. Semi-confluent PC3 cells were incubated in RPMI-0.2%BSA and treated with graded dose of 12(S)-HETE. One day after treatment, the media were collected and VEGF levels in the media were measured using an ELISA kit from R&D Systems (Minneapolis, MN). \*,  $P < 0.05$  when compared to the control.



**Figure 7.** Stimulation of VEGF Promoter Activity by 12(S)-HETE. PC3 cells were transfected with a VEGF promoter luciferase construct, p -1176/+54 and then treated with graded levels of 12(S)-HETE. Cells were harvested 18 hours after treatment and then the luciferase activity was measured by scintillation counting using a kit from Promega Corp. (Madison, WI).

**Task 5.** Determine if 12(S)-HETE has a direct effect on angiogenesis *in vivo* using the CAM assay and the Matrigel implantation assay, Months 12-24:

The studies outlined in the proposed task have been completed. We have used CAM and Matrigel implantation assays to determine whether 12(S)-HETE can stimulate *de novo* angiogenesis. We found that 12(S)-HETE was unable to induce significant angiogenesis either in CAM or Matrigel implantation assay. The results suggest that 12(S)-HETE, while can elicit a number of angiogenic responses such as migration of endothelial cells, but is itself not sufficient to induce significant *de novo* angiogenesis. The results also

suggest that the increased VEGF expression, as observed in **task 2, 3, and 5**, play an important role in the increased angiogenicity of 12-LOX transfected PC-3 cells as observed in **task 1**.

**Task 6.** Determine whether a natural bioactive lipid i.e., 13(S)-HODE can antagonize the effects of 12(S)-HETE in specific aims two through five above, Months 18-30:

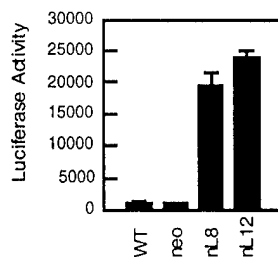
This task has been completed. The premise of this proposed task is that 13(S)-HODE antagonizes the biological function of exogenous 12(S)-HETE (Liu et al., 1995). We found that 13(S)-HODE had minimal inhibitory effect, if any, on the ability of 12-LOX transfected PC-3 cells to stimulate endothelial cell migration as observed in **task 2**. 13(S)-HODE did, however, have slight inhibitory effect on 12(S)-HETE stimulated endothelial cell migration (**task 2**) or VEGF expression in PC-3 cells (**task 4**). Interestingly, 13(S)-HODE did not significantly inhibit the increased VEGF expression in 12-LOX transfected PC-3 cells (task 3). The results suggest that while exogenous 12(S)-HETE has mimicked the biological functions of endogenously produced 12(S)-HETE to some extent, it cannot completely duplicate the function of 12-LOX and its endogenously produced lipid, 12(S)-HETE. Since we did not detect any stimulation of de novo angiogenesis by exogenous 12(S)-HETE, we were unable to study whether 13(S)-HODE antagonizes 12(S)-HETE in stimulation of angiogenesis in vivo. The results from this task provided some insights into the mechanism of 12-LOX as an angiogenic switch.

## Additional Research Progress

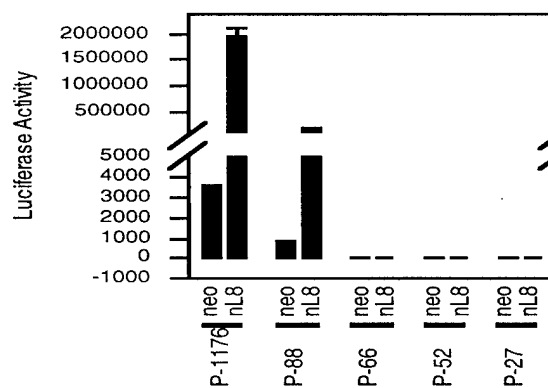
### 1). Transcriptional regulation of VEGF expression by 12-LOX

Since we observed an increase in VEGF production in 12-LOX transfected PC-3 cells when compared to their vector controls, it is of interest to know how 12-LOX regulates VEGF gene expression. The increased VEGF production may contribute, at least partly, to the increased angiogenesis and growth in tumors derived from 12-LOX transfected PC-3 cells. Since the link between 12-LOX and VEGF production in PCa cells has never been reported, it is of importance to study further the molecular mechanisms by which 12-LOX impacts on VEGF expression.

To study whether 12-LOX regulates VEGF expression at the transcriptional level, we transfected neo-control cells (neo- $\alpha$ ) and 12-LOX transfected PC-3 cells (nL-8 or nL-12) with a VEGF promoter luciferase construct (-1176/+54), along with a lacZ control plasmid to normalize transfection efficiency. As shown in **Figure 8**, there was a more than 10-fold increase in VEGF promoter activity in 12-LOX transfected PC-3 cells as compared with their neo-controls.

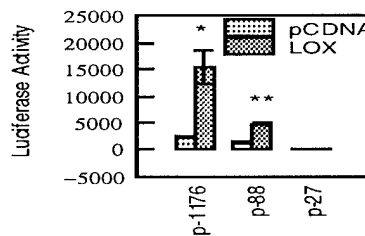


**Figure 8.** Increased VEGF Promoter Activities in 12-LOX Transfected PC-3 Cells.

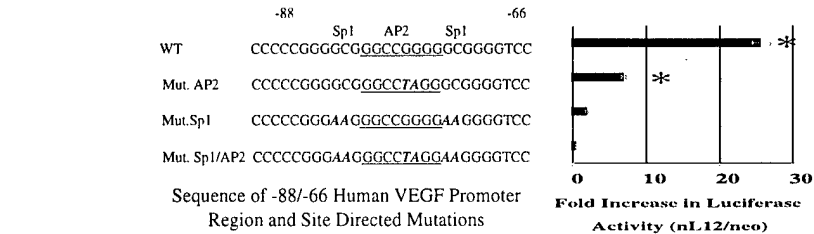


**Figure 9.** Deletion Analysis of VEGF Promoter Activity.

To determine which region of VEGF promoter is 12-LOX-responsive, we transfected neo-control (neo- $\alpha$ ) and 12-LOX transfected PC-3 cells (nL8) with a series of luciferase constructs with different lengths of the VEGF promoter, along with a lacZ control plasmid. As shown in **Figure 9**, deletion of the region between -1176 and -88 significantly reduced, but did not abolish, the increased VEGF promoter activity as observed in nL8. Further deletion of the 23 bp region between -88 and -66 abolished the increased VEGF promoter activity in nL8, indicating the presence of a *cis*-element in this region responsive to 12-LOX. Similar results were obtained with other clones of 12-LOX transfected PC-3 cells (nL2 and nL-12, data not shown).



**Figure 10.** Activation of VEGF Promoter Activity by Co-transfection with a 12-LOX expression construct (LOX). pCDNA, vector control. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  when compared to their respective vector control.



**Figure 11.** Site-Directed Mutation Analysis of the Role of Sp1 and AP2 Recognition Sequences in VEGF Promoter Activity. Left panel, DNA sequence between -88 and -66 of VEGF promoter. Mutated nucleotides in Sp1 recognition sequences (GC to AA) or AP2 binding site (underlined, GG to TA) are shown in italic. Right panel, fold increase of luciferase activities in nL12 when compared to those in neo- $\alpha$ . \*,  $P < 0.05$ , indicating the significant difference in luciferase activities between neo- $\alpha$  and nL12.

Co-transfection of PC-3 parental cells with both VEGF promoter constructs and a 12-LOX expression construct demonstrated a significant increase in p-1176 and p-88, but not p-27, VEGF promoter activities, as compared with their respective pCDNA3.1 vector control (**Figure 10**). The data suggest that 12-LOX stimulates VEGF promoter activity and the observed increase in VEGF promoter activity in 12-LOX transfected PC-3 cells is not due to possible cloning artifacts associated with a particular transfectant clone. The data also demonstrate that the -88 and -27 promoter region of VEGF gene is responsive to 12-LOX, supporting our previous observation that the 23 bp DNA segment between -88 and -66 is required for increased VEGF promoter activity in 12-LOX transfected PC-3 cells.

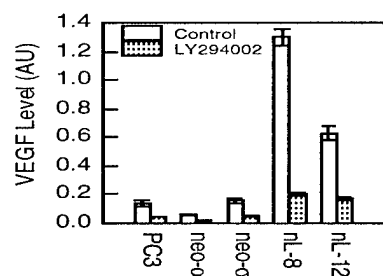
There are one AP2 and two Sp1 binding sites in this 23 bp region. As shown in **Figure 11**, mutation of AP2 decreased, but did not abolish, the stimulation of promoter activity by 12-LOX (from 25 fold to 8 fold increase). In contrast, mutation of two Sp1 binding sites dramatically decreased the stimulation of VEGF promoter activity by 12-LOX in which the increase of VEGF promoter activity in nL12 was no longer statistically significant. Mutation of both AP2 and Sp1 binding sites completely abolished the increased VEGF promoter activity in 12-LOX transfected PC-3 cells. The data suggest that Sp1, and to lesser extent, AP2, are involved in 12-LOX regulation of VEGF promoter activity.

## 2). Cellular signaling in 12-LOX stimulated VEGF gene expression

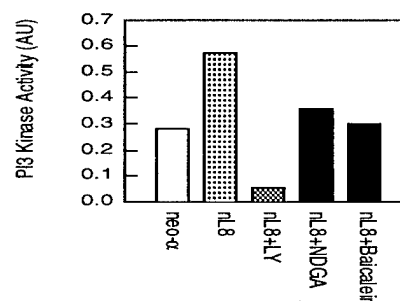
It is known that VEGF expression can be stimulated by a variety of cytokines and growth factors while some cytokines such as IL-10 and IL-13 can inhibit the release of VEGF (Matsumoto et al., 1997). To study the signaling mechanism from 12-LOX leading to VEGF expression in PC-3 cells, we treated 12-LOX transfected PC-3 cells with PD98059, a Mek inhibitor, and LY294002, a PI3 kinase inhibitor, and studied VEGF expression. As shown in **Figure 12**, LY294002 (20  $\mu$ M) reduced VEGF expression in both 12-LOX transfected PC-3 cells (nL-8 and nL-12) and neo-controls (neo- $\alpha$  and neo- $\sigma$ ), as well as in PC-3 parental cell



line. We also found significant reduction of VEGF expression in DU145 cells by LY294002 (data not shown). In contrast, we did not find any effect of PD98059 on VEGF expression in PCa cells (data not shown). The results suggest that PI3 kinase activity is required for VEGF expression in PCa cells as well as in 12-LOX transfected PC-3 cells.



**Figure 12.** Down-regulation of VEGF Expression by PI3 Kinase Inhibitor LY294002.



**Figure 13.** Increased PI3 Kinase Activity in 12-LOX Transfected PC-3 Cells. PI3 kinase activity in p85 immunoprecipitates was measured by incubating with substrate and  $\gamma$ - $^{32}$ P-ATP. Phospholipids were separated by TLC, X-filmed and quantified using a NIH imaging software.

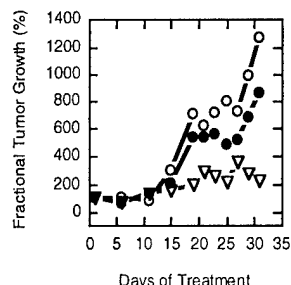
To study whether there is an increase in PI3 kinase activity in 12-LOX transfected PC-3 cells that may mediate the stimulation of VEGF expression, we measured the PI3 kinase activity. As shown in **Figure 13**, there was a 2 fold increase in PI3 kinase activity in 12-LOX transfected PC-3 cells (nL8) and this increase of PI3 kinase was inhibited by pre-treatment of nL8 cells with LOX inhibitors NDGA and baicalein. The data suggest that there is an increase in PI3 kinase activity in 12-LOX transfected PC-3 cells and inhibition of PI3 kinase activities by LY294002 reduced VEGF expression in 12-LOX transfected PC-3 cells and other PCa cells.

### 3). Development of anti-angiogenesis therapy for prostate cancer

There are a number of angiogenesis inhibitors in clinical trials and preclinical development for the treatment of PCa such as CAI (Bauer et al., 1999), TNP-470 (Miki et al., 1998), and VEGF neutralizing antibody (Borgstrom et al., 1998). Most of these angiogenesis inhibitors were developed on the premise that angiogenesis is shared in common by most and perhaps all types of human tumors. Yet, already available evidence indicates that different types of tumor cells may use distinct molecular strategies to activate the angiogenic switch. Human PCa angiogenesis has its uniqueness. This raises the question of whether a single anti-angiogenic therapeutic will suffice to treat all tumor types, or whether an ensemble of such therapeutics will need to be developed, each responding to a distinct program of angiogenesis that has been developed by a specific class of human tumors.

In Phase I of this grant, we found that 12-LOX functions as an “angiogenic switch,” governing PCa angiogenesis and growth. The findings suggest that 12-LOX may be a promising target for developing anti-angiogenesis therapy, at least for those patients whose PCa is 12-LOX positive. Previously, Dr. Honn, in collaboration with Dr. Carl Johnson (Department of Chemistry, Wayne State University) synthesized several classes of hydroxamic acid derivatives, using rational drug design for inhibition of platelet-type 12-LOX (US Patent No. 5,234,933). Compounds were initially screened for inhibition of platelet-type 12-LOX and a select group of them later tested for selectivity. The first lead compound was BMD188 which inhibits 12-LOX with  $IC_{50}$  of 3  $\mu$ M. BMD was initially chosen for study because of its ease of synthesis in bulk, its stability and preliminary pharmacokinetic data indicates a circulating half-life in mouse of 50 hours.

However, BMD188 is relatively insoluble and difficult to formulate. To demonstrate the plausibility of using 12-LOX inhibitors to reduce PCa angiogenesis and tumor growth, we first tested the effect of BMD188 on angiogenesis induced by 12-LOX transfected PC-3 cells in Matrigel plug assay. Athymic mice were injected with  $2 \times 10^6$  12-LOX transfected PC-3 cells suspended in Matrigel. After 24, mice were injected (i.p.) with 20, 60 or 100 mg BMD188 per kg of mouse weight every other day for a total of 4 treatments. A dose dependent inhibition of angiogenesis was observed.



**Figure 15.** Inhibition of Tumor Growth in SCID-Hu Bone by BMD188. Open circle, Solvent control; Filled circle, BMD188 50mg/Kg mouse; Open triangle, BMD188, 100mg/Kg mouse.

Using a SCID-human bone model (Nemeth et al., 1999), we studied whether BMD188 can inhibit the growth of tumors in human bone environment. As shown in **Figure 15**, at 50 or 100 mg BMD188 per kg of mouse weight, significant reduction in tumor growth was observed. Considerable reduction in angiogenesis on the surface of tumor or on the skin around tumors also was observed. The results raise the exciting possibility that inhibition of 12-LOX is a novel approach to inhibit tumor angiogenesis and curb prostate tumor growth.

## KEY RESEARCH ACCOMPLISHMENTS

1. Confirm that 12-LOX functions as an angiogenesis switch governing prostate tumor angiogenesis and growth.
2. Discover a novel link between 12-LOX and VEGF expression in prostate cancer cells.
3. Find a key role of PI3 kinase in 12-LOX stimulated VEGF expression in prostate cancer cells.
4. Demonstrate the plausibility of using 12-LOX inhibitors as anti-angiogenesis compound to inhibit prostate tumor angiogenesis and growth.

## REPORTABLE OUTCOMES

- Research article published.  
Nie, D., G. G. Hillman, T. Geddes, K. Tang, C. Pierson, D. J. Grignon and K. V. Honn. 1998. Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. *Cancer Res.* 58: 4047-4051.
- Research article published.  
Nie, D., K. Tang, C. Diglio and K. V. Honn. 2000. Eicosanoid regulation of angiogenesis: Role of endothelial arachidonate 12-lipoxygenase. *Blood* 95: 2304 - 2311.
- Abstract published.  
Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barrosso, and K.V. Honn. 1999. Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model. *Proc. Amer. Assoc. Cancer Res.* 40: 126.

- Abstract published.  
Nie, D., Y. Chen, K. Tang, K. Hanna, J. Nemeth, G.G. Hillman, M. Cher, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase enhances the metastatic potential of human prostate cancer cells. *Proc. Amer. Assoc. Cancer Res.* 40: 198.
- Abstract.  
Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn. 1999. Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression. *Prostaglandins and Other Lipid Mediators* (In Press).
- Presentation.  
Nie, D., J. A. Nemeth, M.L. Cher, Y. Chen, U. Barroso, and K.V. Honn. "Inhibition of prostate cancer cells by a novel 12-lipoxygenase inhibitor in a human orthotopic bone metastasis model." Poster Presentation at the American Association for Cancer Research 1999 Annual Meeting, Philadelphia, PA, April 10-14, 1999.
- Presentation.  
Nie, D., Y. Chen, K. Tang, K. Hanna, J. Nemeth, G.G. Hillman, M. Cher, D. Grignon, and K.V. Honn. "Arachidonate 12-lipoxygenase enhances the metastatic potential of human prostate cancer cells." Mini-Symposium Presentation at the American Association for Cancer Research 1999 Annual Meeting, Philadelphia, PA, April 10-14, 1999.
- Presentation.  
Nie, D., Y. Chen, K. Tang, G.G. Hillman, D. Grignon, and K.V. Honn.. "Arachidonate 12-lipoxygenase stimulates angiogenesis by up-regulation of vascular endothelial growth factor expression." Oral Presentation Selected by the 6th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases, Boston, MA, Sept. 12 -15, 1999.
- Development of animal models: Yes.  
We developed a Matrigel implantation assay model for study of PCa cell induced angiogenesis.
- Funding applied or obtained: Yes  
Based on the above-described findings, PI was recognized by a CaPCURE award in 1999, in the amount of \$75,000, to develop prostate-targeting 12-LOX inhibitors for the treatment of prostate cancer.

## CONCLUSIONS

The research progress made in Phase I of Award# DAMD17-98-1-8502 indicates that 12-LOX function as a novel "angiogenic switch" governing prostate cancer angiogenesis and tumor growth. We found that 12-LOX increased the angiogenic potential of PCa cells by stimulating the production of VEGF, in addition to its arachidonate product 12(S)-HETE. The link between 12-LOX, a free fatty acid metabolizing enzyme, and VEGF, a putative angiogenic factor, is both novel and exciting, providing significant insights into our understanding of the regulation of VEGF expression during PCa progression. The study also found the transcriptional regulation of VEGF expression by 12-LOX and by its lipid product, 12(S)-HETE. The study also demonstrated the plausibility of using 12-LOX inhibitors such as BMD188 to inhibit prostate tumor angiogenesis and growth.

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## LIST OF APPENDICES

1. Nie, D., G.G. Hillman, T. Geddes, K. Tang, C. Pierson, D. J. Grignon, and K. V. Honn. (1998). Platelet-type 12-lipoxygenase in a human prostate carcinoma stimulates angiogenesis and tumor growth. *Cancer Res.* 58, 4047 – 4051.
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# Platelet-Type 12-Lipoxygenase in a Human Prostate Carcinoma Stimulates Angiogenesis and Tumor Growth<sup>1</sup>

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## Abstract

Previously, we found a positive correlation between the expression of platelet-type 12-lipoxygenase (12-LOX) and the progression of human prostate adenocarcinoma (PCa; Gao *et al.*, Urology, 46: 227-237, 1995). To determine the role of 12-LOX in PCa progression, we generated stable 12-LOX-transfected PC3 cells, which synthesize high levels of 12-LOX protein and 12(S)-hydroxyicosatetraenoic acid metabolite. *In vitro*, 12-LOX-transfected PC3 cells demonstrated a proliferation rate similar to neo controls. However, following s.c. injection into athymic nude mice, 12-LOX-transfected PC3 cells formed larger tumors than did the controls. Decreased necrosis and increased vascularization were observed in the tumors from 12-LOX-transfected PC3 cells. Both endothelial cell migration and Matrigel implantation assays indicate that 12-LOX-transfected PC3 cells were more angiogenic than their neo controls. These data indicate that 12-LOX stimulates human PCa tumor growth by a novel angiogenic mechanism.

## Introduction

The growth and metastasis of solid tumors are dependent upon the ability of tumor cells to induce angiogenesis (1). Angiogenesis, the formation of new blood vessels from preexisting ones, involves endothelial cell proliferation, motility, and differentiation. Tumor cells can secrete a variety of angiogenic factors, such as basic fibroblast growth factor and vascular endothelial growth factor, to stimulate angiogenesis (2). Tumor cells also produce angiogenesis inhibitors such as thrombospondin and angiostatin to control angiogenesis (2). The balance between angiogenesis stimulators and inhibitors determines the angiogenicity of tumor cells (2). In human PCa,<sup>3</sup> the level of vascularization positively correlates with tumor stage (3-5). Inhibition of angiogenesis by linomide or TNP-470 potently inhibits PCa growth and metastasis by causing necrosis and apoptosis in tumors (6, 7). Although various potential angiogenesis factors have been identified in prostate cancer (8), it is still unclear by which process PCa cells become angiogenic. We have previously detected the expression of platelet-type 12-LOX in human PCa and demonstrated a correlation between 12-LOX mRNA expression and pathological stage (9). Platelet-type 12-LOX uses only arachidonic acid as substrate and forms 12(S)-HETE exclusively (10). Here, we have examined the function of 12-LOX on PCa tumor growth. Our data dem-

onstrate that 12-LOX has no detectable effect on PCa cell growth *in vitro* but stimulates PCa tumor growth *in vivo*. This effect of 12-LOX on tumor growth is closely related to increased angiogenesis. Both *in vitro* and *in vivo* angiogenesis assays suggest that PCa cells expressing high levels of 12-LOX are more angiogenic than those expressing no or low levels of 12-LOX. Our results provide a novel function for platelet-type 12-LOX in PCa progression.

## Materials and Methods

**Cell Culture.** Rat angiogenic endothelial cell line RV-ECT (a gift from Dr. Clement Diglio, Department of Pathology, Wayne State University) was maintained in DMEM with 10% FBS (11). The cells were used between passage numbers 29 and 34. The human prostate carcinoma cell line PC3 was originally purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 with 10% FBS. All culture reagents were purchased from Life Technologies, Inc.

**Stable Transfection of PC3 Cells and Characterization.** Passage 28 PC3 cells were cotransfected using a Lipofectin reagent (Life Technologies, Inc.) with a pCMV-platelet-type 12-LOX construct (a gift from Dr. Collin Funk, Center for Experimental Therapeutics, University of Pennsylvania; Ref. 10), and pCMV-*neo*, which encodes a neomycin-resistant protein. PC3 cells transfected with pCMV-*neo* were used as controls. Transfectants were selected using 1 mg/ml geneticin (G418) in RPMI with 10% FBS and then cloned using a limiting dilution method in 96-well plates. The cloned transfectants were propagated and characterized for 12-LOX mRNA expression by Northern blot and 12-LOX protein expression by Western blot. Human epidermoid carcinoma A431 cells that express 12-LOX (12) were used as a positive control. The probe used in Northern blot was the 12-LOX cDNA from pCMV 12-LOX construct. Rabbit 12-LOX polyclonal antibody used in Western blot was purchased from Oxford Biomedical Inc. (Oxford, MI). Actin antibody was from Amersham (Arlington Heights, IL). The synthesis of 12(S)-HETE by 12-LOX transfectants was determined using a RIA kit from Perspective Diagnostics (Cambridge, MA) according to the manufacturer's instructions.

**In Vitro Proliferation Assay.** To study the growth kinetics of PC3 transfectants in culture,  $2 \times 10^3$  cells per well were seeded in 96-well culture plate. The number of viable cells at intervals of 48 h was assessed using an MTS cell proliferation assay kit (Promega Corp, Madison, MI). The  $A_{490\text{ nm}}$  readings 2-3 h after plating were used as baselines. The number of cells was expressed as the percentage of increase from the  $A_{490\text{ nm}}$  baselines.

**Animal Model and Histochemical Studies.** A total of  $4 \times 10^6$  12-LOX-transfected PC3 cells or neo control cells in 200  $\mu$ l of HBSS were injected s.c. into the right flank of 4-6-week-old male BALB/c nude mice (obtained from University of South Florida, Tampa, FL). The resulting tumors were measured using a vernier caliper, and tumor volume was calculated using the formula: (width)<sup>2</sup>  $\times$  length  $\times$  0.5 (7). Six to 7 weeks after injection, mice were sacrificed, and the tumors were resected and photographed under an SP SZ-4060 stereomicroscope (Olympus America, Melville, NY). Tumors were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections (5  $\mu$ m) were prepared for histology staining. Sections were stained with H&E to examine the presence of necrosis. The assessment of tumor necrotic area was performed for a total of 10 HPFs per tumor using a double-blind approach.

CD31 staining was used to assess tumor vascularization. Immunohistochemical staining for CD31 (DAKO Corp.; dilution, 1:20) was performed

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<sup>3</sup> The abbreviations used are: PCa, prostate adenocarcinoma; 12-LOX, 12-lipoxygenase; 12(S)-HETE, 12(S)-hydroxyicosatetraenoic acid; RV-ECT, rat vascular endothelial cells-tube forming; FBS, fetal bovine serum; HPF, high-power field.

using a standard avidin-biotin complex-immunoperoxidase procedure. The slides were counterstained with hematoxylin. The vascularity was assessed qualitatively on the basis of overall vessel organization and quantitatively by microvessel density. A total of 10 fields per tumor were evaluated for both microvessel density and vessel organization. The microvessel density was indicated by the average number of vessels crossing an arbitrary line across one HPF field. The rating of vessel organization was performed according to the following scale: 0, disorganized, staining randomly distributed; 1, intermediate, vessel-like structures formed; and 2, highly organized, vessels structured and organized.

**Endothelial Cell Migration Assay.** For the cell migration assay, RV-ECT endothelial cells were harvested by trypsinization and resuspended in RPMI with 10% FBS, and  $5 \times 10^5$  cells in 0.5 ml were plated on the top chamber of a modified Boyden chamber (Becton Dickinson, Bedford, MA). Then, 1 ml of RPMI-10% FBS medium conditioned from PC3 or various transfectant cultures or fresh medium with 12(S)-HETE was added in triplicate into the lower chamber. After 4 h of incubation, the cells on the top side of the transwell membrane were removed with cotton swabs. The membrane was then cut out, fixed in a quick-fix solution, double-stained, and mounted for observation and counting. Usually, 12 fields ( $\times 100$ ) representing two perpendicular cross-lines of each membrane were counted.

**Matrigel Implantation Assay for Tumor Cell-induced Angiogenesis.** The Matrigel implantation assay was performed as described by Ito *et al.* (13) with the following modifications. Matrigel (Becton Dickinson, Bedford, MA; 0.4 ml premixed with  $2 \times 10^6$  PC3 12-LOX transfectant or neo control cells) was injected s.c. into nude mice (four mice per group). Mice were sacrificed 12 days after injection and dissected to expose the implants for recording.

## Results

**Generation of PC3 Transfectants That Constitutively Synthesize 12-LOX and 12(S)-HETE.** To determine the function of 12-LOX in PCa progression, PC3 cells were transfected with a platelet-type 12-LOX cDNA construct. Stable transfectants were cloned and named the nL series. Several stable transfectants (neo series) isolated from PC3 cells transfected with pCMV-*neo* were used as controls. Northern blot analyses of transfectant clones show that the levels of 12-LOX mRNA were increased in various nL clones, compared to the neo controls or wild-type PC3 (Fig. 1A). The 12-LOX mRNA levels in various nL clones were higher than in A431, a cell line that constitutively expresses 12-LOX (12). 12-LOX-transfected PC3 cells also had higher levels of 12-LOX protein than neo controls or wild-type PC3, as revealed by Western blot analysis (Fig. 1B). Among the various clones analyzed, nL-2, nL-8, nL-11, and nL-12 expressed 12-LOX at the highest levels. We also found that 12-LOX-transfected PC3 clones nL-2, nL-8, and nL-12 synthesized 6–10-fold more 12(S)-HETE than the neo control or wild-type PC3 cells (Fig. 1C), indicating that 12(S)-HETE biosynthesis was greatly enhanced in 12-LOX-transfected PC3 cells.

**12-LOX Transfectants Have an *in Vivo* but not an *in Vitro* Growth Advantage.** *In vitro*, the growth rates of several 12-LOX transfectant clones were similar to those of neo controls and wild-type PC3 cells (Fig. 2A), with an approximate doubling time of 36 h. However, following s. c. injection into nude mice, 12-LOX-transfected PC3 cells (nL-2 and nL-12) grew faster and formed larger tumors than did neo controls (neo- $\sigma$  and neo- $\alpha$ ; Fig. 2B). As shown in Fig. 2C, tumors derived from 12-LOX-transfected PC3 cells were larger than those obtained from neo controls, indicating that 12-LOX-transfected PC3 cells had an *in vivo* growth advantage compared to neo controls or wild-type PC3 cells. Similar results were obtained with an additional 12-LOX transfectant clone tested (nL-8; data not shown). Assessment of tumor necrosis from H&E-stained tumor sections revealed that tumor necrosis was significantly reduced in the tumors derived from 12-LOX-transfected PC3 cells ( $P < 0.05$  by Student's *t* test), whereas 12.1% of tumor area of neo- $\sigma$  tumors were necrotic ( $n = 7$ ; range, 5–35%), only 1.9% of tumor area in the nL-12

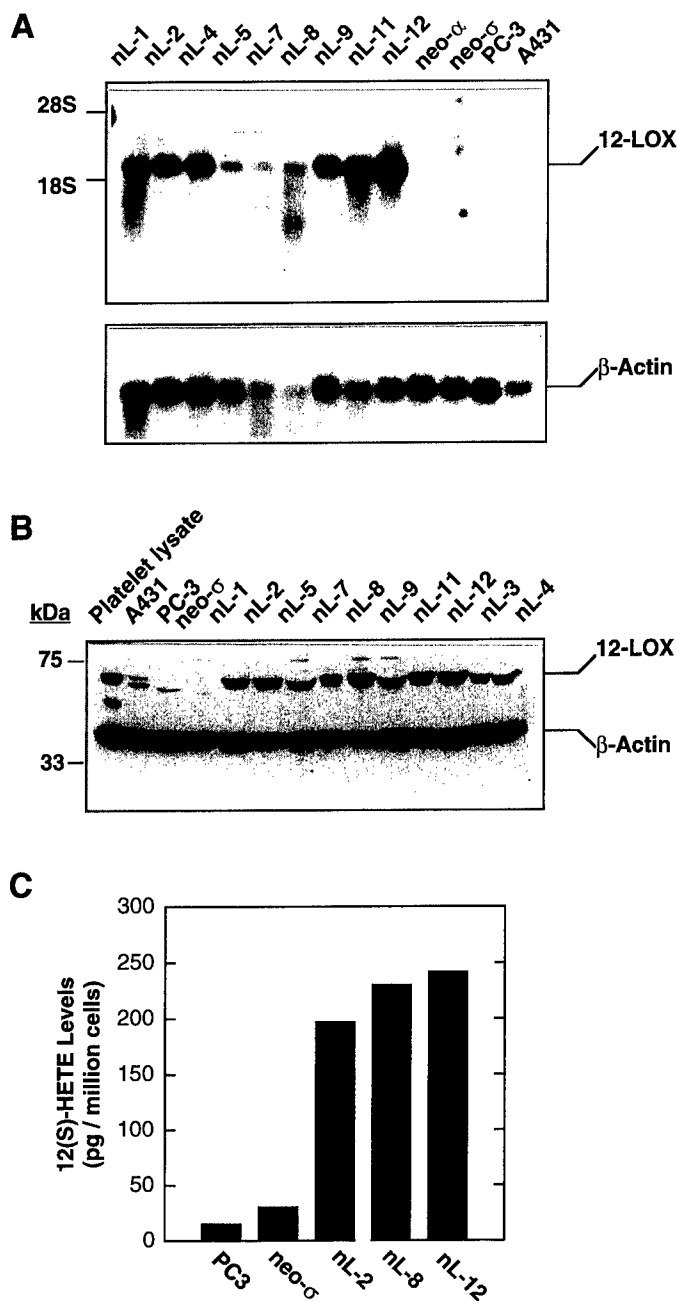


Fig. 1. Generation of PC3 transfectants synthesizing high levels of 12-LOX and 12(S)-HETE. The transfection of PC3 cells and the cloning of stable transfectants were performed as described in "Materials and Methods." A, Northern blot analysis of 12-LOX mRNA levels in various clones of PC3 12-LOX transfectants. Top, blot probed with 12-LOX cDNA; bottom, blot probed with actin cDNA as the loading control. B, Western blot analysis of 12-LOX protein expression in various clones of PC3 12-LOX transfectants. The blot was probed with a 12-LOX polyclonal antibody and actin antibody. C, 12(S)-HETE levels in various 12-LOX transfectants. The levels of 12(S)-HETE in total cell lysates were measured using RIA and were normalized to cell number and expressed as pg of 12(S)-HETE/ $1 \times 10^6$  cells.

clone was necrotic ( $n = 8$ ; range, 0–10%). A significant decrease in tumor necrosis was also observed in the tumors derived from 12-LOX transfectants nL-2 and nL-8, compared to neo- $\alpha$  (data not shown), suggesting that the increased tumor growth by 12-LOX transfectants is mainly due to the reduction of tumor necrosis.

**Increased Angiogenesis in the Tumors from 12-LOX Transfectants.** Because angiogenesis plays an important role in tumor growth by influencing tumor necrosis and apoptosis (2), we studied whether the increased tumor growth by 12-LOX transfectant is angiogenesis

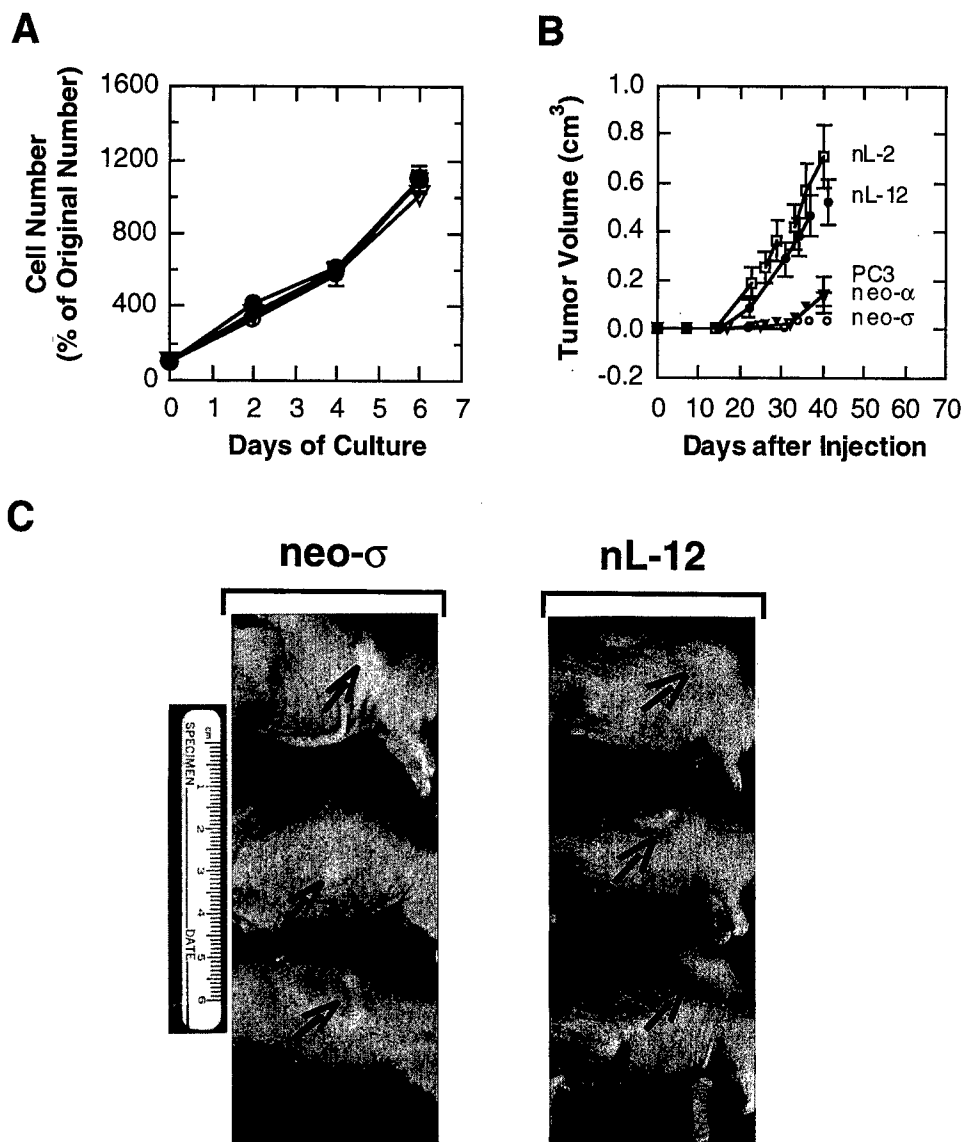


Fig. 2. 12-LOX transfectants have an *in vivo* but not *in vitro* growth advantage. **A**, growth kinetics of PC3 transfectants in culture. Cell proliferation of various transfectants was measured as described in "Materials and Methods." Shown here are the growth curves of PC3 wild type ( $\circ$ ), neo- $\sigma$  ( $\bullet$ ), nL-8 ( $\nabla$ ), and nL-12 ( $\blacktriangle$ ). Data points, means of six determinations; bars, SE. Other clones such as nL-2 and neo- $\alpha$  also had similar growth kinetics (data not shown). **B**, growth kinetics of the tumors derived from 12-LOX transfectants and neo controls. Data points, mean volumes of eight tumors for nL-12 ( $\bullet$ ) and neo- $\sigma$  ( $\circ$ ), five tumors for nL-2 ( $\square$ ) and neo- $\alpha$  ( $\nabla$ ), and six tumors for PC3 wild type ( $\blacktriangledown$ ); bars, SE. **C**, mice with tumors from 12-LOX transfectants or from neo control. Left, three mice with tumors from neo- $\sigma$  (arrows); right, three mice bearing tumors from 12-LOX-transfected PC3 cells (nL-12; arrows).

dependent. We found significant vascularization in tumors derived from 12-LOX-transfected PC3 cells, whereas the neo control tumors showed little vessel penetration (Fig. 3A). Immunostaining with CD31 antibody, which detects the presence of endothelial cells, showed that the vascular networks in tumors derived from nL-12 were sinusoidal in pattern and well developed in structure (Fig. 3B, right). In contrast, in neo control tumors, endothelial cells were present but were randomly distributed and did not form an organized vascular network (Fig. 3B, left). There were fewer vessels in neo- $\sigma$  tumors than in nL-12, as suggested by microvessel density (Fig. 3C). The assessment of the vessel organization demonstrated that the majority of vessels in the tumors derived from 12-LOX-transfected PC3 cells were highly organized, whereas in those from neo- $\sigma$ , they showed a disorganized to intermediate pattern (Fig. 3D). In tumors derived from nL-2 and nL-8, we also observed a similar increase in angiogenesis when compared to neo- $\alpha$  (data not shown).

**Increased Angiogenicity of 12-LOX Transfectants.** The increased angiogenesis in the tumors generated from 12-LOX-transfected PC3 cells raises the question of whether the observed increase in angiogenesis is the cause or a consequence of the increased tumor growth. To address this issue, we first assayed the conditioned culture medium of PC3 12-LOX-transfected PC3 cells or neo controls for

their ability to stimulate endothelial cell migration. As shown in Fig. 4A, the medium from the 12-LOX-transfected PC3 cells induced more RV-ECT migration than did the medium from neo controls. Under similar assay conditions, 12(S)-HETE itself also stimulated RV-ECT migration at nanomolar levels (Fig. 4B). The increased angiogenicity of 12-LOX transfectants was confirmed by the Matrigel implantation assay. As shown in Fig. 4C, within 12 days, 12-LOX-transfected PC3 cells (nL-12) in Matrigel induced massive angiogenesis, indicated by the accumulation of blood in the gel, compared to the neo control (neo- $\sigma$ ). The results clearly illustrate that the 12-LOX-transfected PC3 cells are more angiogenic than their neo controls.

## Discussion

Here, we found that the increased expression of 12-LOX in human PCa cells stimulates prostate tumor growth by enhancing their angiogenicity. The findings have significant bearing on the regulation of PCa progression because, in patients diagnosed with prostate carcinoma, some tumors are extremely malignant, with rapid progression, whereas others are localized and dormant for many years. Exploration of the mechanism underlying the transition from latent to rapidly growing PCa will provide useful information for PCa management.

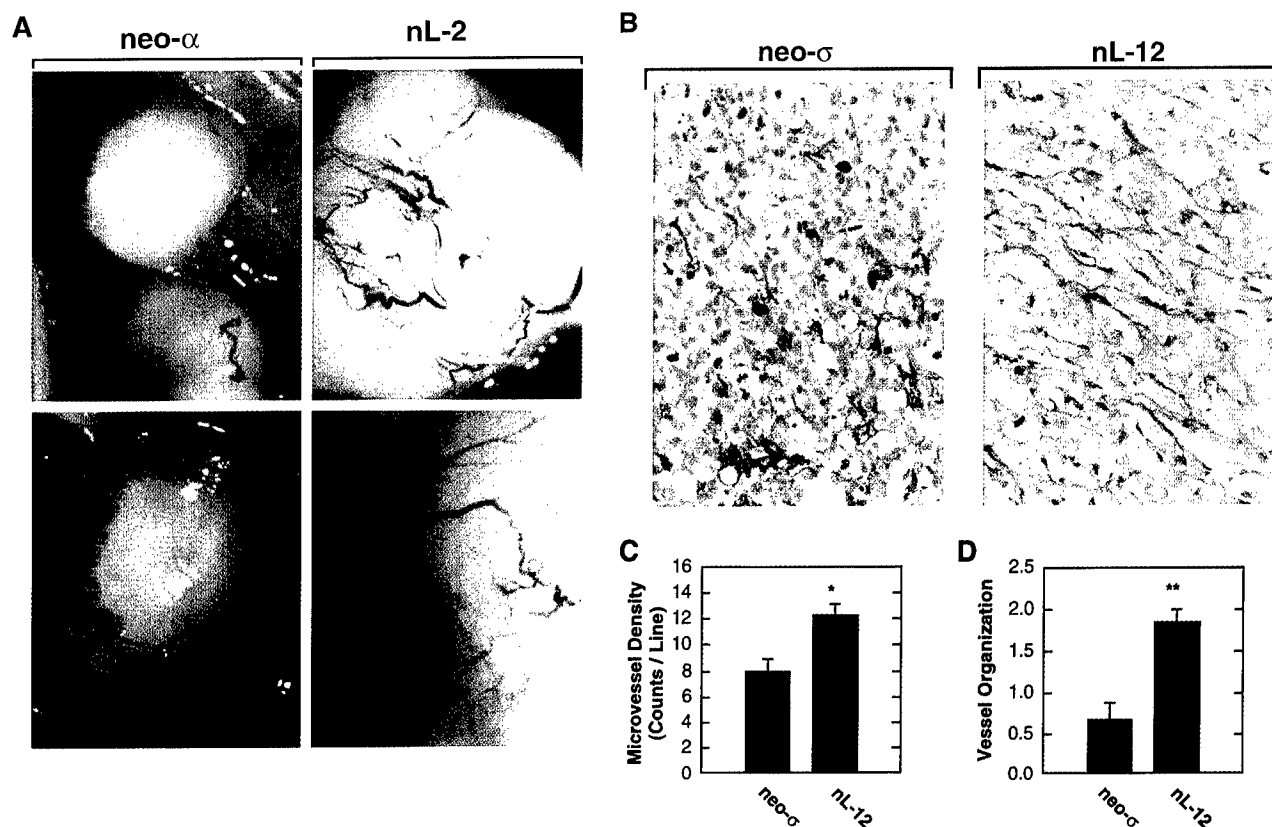


Fig. 3. Increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells. *A*, tumor morphology. *Left*, two tumors from neo- $\alpha$ ; *right*, two tumors from 12-LOX-transfected PC3 cells (nL-2).  $\times 8$ . *B*, CD31 immunostaining. *Brown*, positive staining. *Left*, control tumor. Note the scattered vascular spaces which are randomly distributed and do not form a structured vascular network. *Right*, tumor from 12-LOX-transfected PC3 cells. Note the numerous vascular channels showing a highly organized sinusoidal pattern surrounding small nests of tumor cells.  $\times 250$ . *C*, microvessel density. *Columns*, microvessel densities, expressed as the average number of vessel-like structures crossing an arbitrary line in one HPF; *bars*, SE. Note the significant increase in microvessel density in the tumors derived from nL-12 ( $n = 7$ ) as compared in those of neo- $\sigma$  ( $n = 7$ ; \*,  $P < 0.05$  by Student's  $t$  test). *D*, organization of intratumoral blood vessels. The vessel organization was scored as described in "Materials and Methods." *Columns*, mean scores of tumors derived from nL-12 ( $n = 7$ ) and neo- $\sigma$  ( $n = 7$ ); *bars*, SE (\*\*,  $P < 0.01$  by Student's  $t$  test).

Our observations here, together with our previous demonstration of the correlation between 12-LOX expression and PCa progression in clinical samples (9), suggest that 12-LOX may play a critical role in the progression of human PCa.

The increased tumor growth observed with 12-LOX-transfected PC3 cells is due to the reduction in tumor necrosis as a result of increased angiogenesis. The increased 12-LOX levels in PC3 cells did not confer a growth advantage *in vitro*, suggesting that 12-LOX overexpression does not have direct effect on PC3 cell growth and that the growth advantage of 12-LOX transfectants *in vivo* is due to the host environment. This tumor-host interaction based mechanism is supported by the observed increase in angiogenesis in the tumors from 12-LOX-transfected PC3 cells. Because angiogenesis is required for tumor expansion, the lack of or inhibition of angiogenesis has been demonstrated to induce tumor cell necrosis and apoptosis, thereby limiting tumor growth in PCa (2, 6–7). Indeed, histological analysis revealed that the tumors derived from neo controls had increased necrosis, suggesting that it is the insufficient vascularization that limited the growth of the neo control tumors.

The increased angiogenesis in the tumors from 12-LOX-transfected PC3 cells is at least partly due to their increased angiogenicity. 12-LOX-transfected PC3 cells have increased ability to stimulate endothelial cell migration *in vitro* and neovascularization of Matrigel *in vivo*, compared to their neo controls. The angiogenicity of tumor cells is controlled by the balance between stimu-

lators and inhibitors of angiogenesis (2). Therefore, it will be interesting to determine how 12-LOX up-regulates the angiogenicity of PCa cells. One explanation is that 12-LOX or 12(S)-HETE may increase the angiogenicity of tumor cells by influencing the expression of angiogenic or angiostatic molecules. An alternative interpretation is that 12(S)-HETE may directly alter the balance in favor of angiogenic factors due to its proangiogenic nature. This is supported by our finding here that 12(S)-HETE stimulated endothelial cell migration at nanomolar levels and previous reports showing that 12(S)-HETE stimulated endothelial cell proliferation (14), retraction (15), and adhesion and that it increased the surface expression of integrin  $\alpha_v\beta_3$  in both macro- and microvascular endothelial cells (16). It is noteworthy that integrin  $\alpha_v\beta_3$  is predominantly associated with angiogenic blood vessels (17) and plays an essential role in human cancer angiogenesis (18). Thus, 12(S)-HETE may directly increase the angiogenicity of PCa cells by stimulating angiogenesis or by eliciting several proangiogenic responses that can be additive or synergistic to effects from other angiogenic factors produced by PCa cells because different factors have their own distinct effects on the process of angiogenesis (19). Studies are ongoing to determine whether increased 12-LOX expression in PCa cells influences the gene expression of angiogenic factors and whether 12(S)-HETE can stimulate angiogenesis alone or by its additive or synergistic interaction with other putative angiogenic factors.



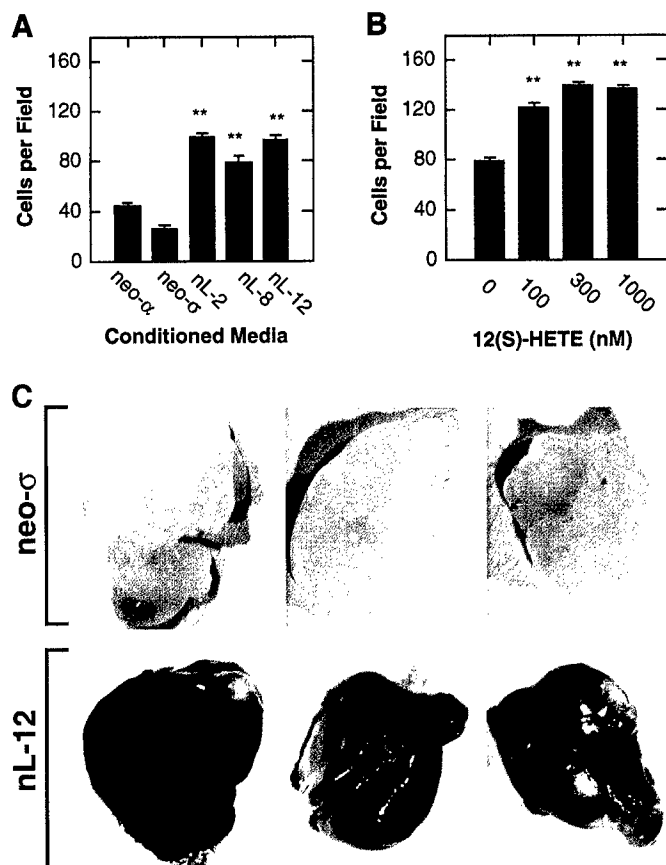


Fig. 4. Increased angiogenicity of 12-LOX-transfected PC3 cells. **A**, stimulation of endothelial cell migration by the conditioned medium from 12-LOX transfectants. The conditioned media were harvested after 24 h of culture and used for migration assay as described in "Materials and Methods." Columns, average numbers of cells migrated per field; bars, SE (\*\*,  $P < 0.01$  by Student's  $t$  test). **B**, 12(S)-HETE stimulates endothelial cell migration. The migration assay was performed essentially as described in **A** except that media with various levels of 12(S)-HETE, instead of the conditioned media, were placed into the lower chamber. Columns, means; bars, SE (\*\*,  $P < 0.01$  by Student's  $t$  test). **C**, induction of angiogenesis in Matrigel by 12-LOX transfectants. *Top*, three Matrigel implants premixed with  $2 \times 10^6$  neo-σ cells. Note the vessel penetration into the gel was minimal, with little blood accumulated in the gel. *Bottom*, in contrast, the Matrigel premixed with  $2 \times 10^6$  12-LOX transfectant (nL-12) demonstrates considerable blood accumulation.

One final point concerns the expression of 12-LOX during human PCa progression. If the effects of 12-LOX on PCa tumor growth and angiogenesis just described are of physiological significance, it should be expected that PCa cells express 12-LOX. This has, in fact, been observed *in vivo*, where the expression of 12-LOX has been positively correlated with tumor stage (9). The question of how 12-LOX expression is up-regulated in PCa cells is currently being explored.

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## Eicosanoid regulation of angiogenesis: role of endothelial arachidonate 12-lipoxygenase

Daotai Nie, Keqin Tang, Clement Diglio, and Kenneth V. Honn

**Angiogenesis, the formation of new capillaries from preexisting blood vessels, is a multistep, highly orchestrated process involving vessel sprouting, endothelial cell migration, proliferation, tube differentiation, and survival. Eicosanoids, arachidonic acid (AA)-derived metabolites, have potent biologic activities on vascular endothelial cells. Endothelial cells can synthesize various eicosanoids, including the 12-lipoxygenase (LOX) product 12(S)-hydroxyeicosatetraenoic acid (HETE). Here we demonstrate that endogenous 12-LOX is involved in endothelial cell angiogenic responses. First, the 12-LOX**

**inhibitor, N-benzyl-N-hydroxy-5-phenylpentanamide (BHPP), reduced endothelial cell proliferation stimulated either by basic fibroblast growth factor (bFGF) or by vascular endothelial growth factor (VEGF). Second, 12-LOX inhibitors blocked VEGF-induced endothelial cell migration, and this blockage could be partially reversed by the addition of 12(S)-HETE. Third, pretreatment of an angiogenic endothelial cell line, RV-ECT, with BHPP significantly inhibited the formation of tubelike/cordlike structures within Matrigel. Fourth, overexpression of 12-LOX in the CD4 endothelial cell line significantly**

**stimulated cell migration and tube differentiation. In agreement with the critical role of 12-LOX in endothelial cell angiogenic responses in vitro, the 12-LOX inhibitor BHPP significantly reduced bFGF-induced angiogenesis in vivo using a Matrigel implantation bioassay. These findings demonstrate that AA metabolism in endothelial cells, especially the 12-LOX pathway, plays a critical role in angiogenesis. (Blood. 2000;95:2304-2311)**

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### Introduction

The formation of new capillaries from preexisting vessels, a process termed angiogenesis, is tightly regulated in physiologic processes such as embryonic development, wound repair, and hypertrophy of normal organs. In contrast, persistent unregulated angiogenesis underscores many pathologic conditions, such as tumor growth and metastasis, diabetic retinopathy, atherosclerosis, and chronic inflammation. Angiogenesis is a complex process involving an extensive interplay between cells, soluble factors, and extracellular matrix molecules that culminate in the proliferation, migration, and tube differentiation of endothelial cells.<sup>1</sup> A plethora of angiogenesis regulators such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) can elicit various angiogenic responses from endothelial cells.<sup>2</sup> An understanding of endothelial cell metabolism and the signaling that underlies angiogenesis is important because it provides potential therapeutic targets to inhibit or enhance angiogenesis.

12-lipoxygenases (12-LOX) are a family of isozymes that belong to the LOX superfamily. These enzymes catalyze the stereospecific oxygenation of arachidonic acid (AA) to form 12(S)-hydroperoxyeicosatetraenoic acid (HPETE) and 12(S)-hydroxyeicosatetraenoic acid (HETE). At least 3 types of 12-LOX have been well characterized: platelet-type, leukocyte-type, and epidermal 12-LOX.<sup>3</sup> Platelet-type 12-LOX exclusively uses AA released from glycerophospholipid pools to synthesize 12(S)-HPETE and 12(S)-HETE, whereas leukocyte-type 12-LOX can

also synthesize 15(S)-HETE and 12(S)-HETE. In addition to leukocytes and platelets, the expression of 12-LOX isozymes has been detected in various types of cells, such as smooth muscle cells,<sup>4</sup> keratinocytes,<sup>5</sup> endothelial cells,<sup>4,6</sup> and tumor cells. Elevated 12-LOX activity has been implicated in hypertension,<sup>7</sup> vaso-occlusion in sickle cell disease,<sup>8</sup> inflammation,<sup>9</sup> thrombosis,<sup>10</sup> and mouse skin tumor development.<sup>5</sup> In human prostate carcinoma, the level of 12-LOX expression has been correlated with tumor stage.<sup>11</sup> Along this line, we recently demonstrated that the overexpression of platelet-type 12-LOX in human prostate cancer PC3 cells stimulated tumor growth by elaborating tumor angiogenesis.<sup>12</sup>

In endothelial cells, it has been shown that 12-LOX activity is required for serum- and bFGF-stimulated endothelial cell proliferation<sup>13,14</sup> and for minimally modified low-density lipoprotein-induced monocyte binding to endothelial cells.<sup>15</sup> It has been shown that 12(S)-HETE can directly stimulate endothelial cell mitogenesis,<sup>13,16</sup> migration,<sup>12</sup> and surface expression of  $\alpha_v\beta_3$  integrin.<sup>17-20</sup> Because endothelial cell proliferation and migration and increased levels of surface  $\alpha_v\beta_3$  integrin<sup>21-23</sup> are involved in angiogenesis, these observations prompted us to investigate the functional role of endothelial 12-LOX in angiogenesis. Here we report that endothelial 12-LOX activity is required for endothelial cell proliferation, migration, and tube differentiation in vitro and angiogenesis in vivo. This study suggests the importance of arachidonic acid metabolism in endothelial cell signaling as it relates to angiogenesis.

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## Materials and methods

### Inhibitors

BHPP, a 12-LOX inhibitor as previously described,<sup>24</sup> was a generous gift from Biomide (Grosse Pointe Farms, MI). The  $IC_{50}$  for BHPP to inhibit platelet-type 12-LOX activity in tumor cells is approximately 0.2 to 1  $\mu\text{mol/L}$ , depending on cell type.<sup>24,25</sup> BHPP does not appreciably inhibit 5(S)-HETE or 15(S)-HETE synthesis in highly metastatic B16a cells.<sup>25</sup> Using recombinant enzymes, the  $IC_{50}$  of BHPP for the murine platelet-type 12-LOX is 0.8  $\mu\text{mol/L}$  with arachidonic acid as a substrate. The rank of selectivity of BHPP for lipoxygenase inhibition is murine platelet-type 12-LOX > murine epidermis-type 12-LOX > human 5-LOX >> murine leukocyte 12-LOX >> rabbit 15-LOX-1 (Furstenberger G, personal communication). Because of its selectivity toward the platelet-type 12-LOX, BHPP was extensively used in the current study. Other inhibitors for AA metabolism—5-, 8-, 11-, and 14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), 5-LOX-activating protein (FLAP) inhibitor MK886, and cyclooxygenase (COX) inhibitor indomethacin—were purchased from Calbiochem (San Diego, CA). Their sites of action are illustrated in Figure 1.

### Cell culture

The cord-forming angiogenic endothelial cell line RV-ECT was isolated from the RV-EC cell line established from rat brain resistance vessels as previously described.<sup>26</sup> The mouse capillary endothelial cell line CD4 was originally established from mouse lung capillary blood vessels.<sup>27</sup> Both the RV-ECT and the CD4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) and human foreskin dermal microvascular endothelial cells (HMVEC) were purchased from Clonetics (San Diego, CA), multiplied in EGM-2, and used from passages 4 to 10.

### Detection of 12-LOX expression by reverse transcription–polymerase chain reaction

Total RNA was isolated from semiconfluent (70% to 80%) endothelial cells using Tri-reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendations. After total RNA was isolated, it was further washed with 2 mol/L LiCl/5 mmol/L EDTA to reduce possible contamination from genomic DNA. Total RNA was reverse transcribed with oligo dT using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD). To detect the expression of platelet-type 12-LOX in human endothelial cells, nested polymerase chain reaction (PCR) was conducted using primers located in different exons of the human platelet-type 12-lipoxygenase to ensure the differentiation of PCR products from cDNA or genomic DNA as previously described.<sup>11</sup> The size of the first-round PCR product was approximately 590 bp and the second-round PCR approximately 190 bp. Negative controls with no reverse transcriptase added were also used to detect the possibility of false-positive signals. The PCR products were analyzed using 2% agarose gel.

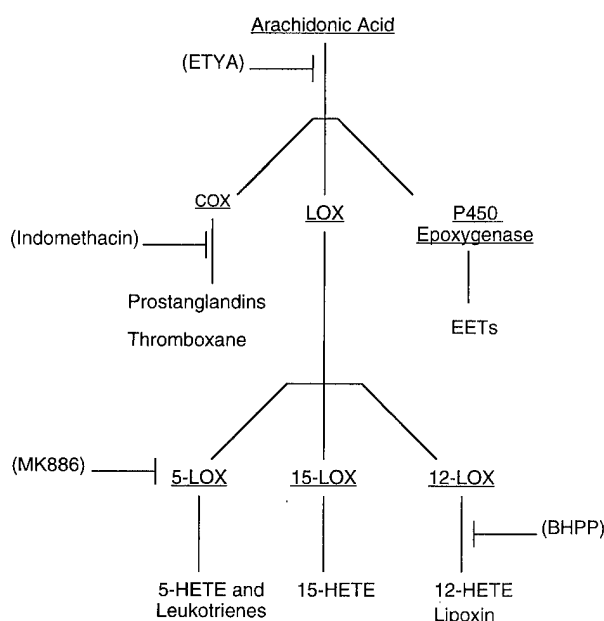
To confirm further the expression of platelet-type 12-LOX in rat RV-ECT endothelial cells, primers were designed based on the partial sequence of rat platelet-type 12-LOX obtained from rat Walker 256 cells<sup>24</sup> because full-length rat platelet-type 12-LOX has not been cloned or sequenced. Total RNA was isolated using tri-reagent, and the cDNA sequence was synthesized using adapter primer according to the standard protocol from Gibco BRL's 3' RACE kit (Rapid Amplification of cDNA Ends kit; #18,73-027; Life Technologies). Three different combinations of primers were used for nested PCR. The first combination yielded the final product of 111 bp with the first-round PCR primer set of AGACAATAGCAGCAGACT (1 U) and TAGACGGTTCAGCTT (137 L) and the second-round primer set of TGACCTCCCTCAAACAT (26 U) and CTCAGGTATAAACA (119 L). The second combination yielded the final

product of 62 bp using AGACAATAGCAGCAGACT (1 U) and CTCAGGTATAAACA (119 L) as the first-round primer set and TGACCTCCCTCAAACAT (26U) and TCAGCGTCCATTCTAAGT (70L) as the second PCR primer set. The expected product size of the third combination was 88 bp using CAGGAGACAATGCTTTTGGAC (LOS2) and GAA-CACTCATCATCCTGCC (LO-AS2) as the first PCR primer set and AGACAATAGCAGCAGACT (1 U) and TCAGCGTCCATTCTAAGT (70 L) as the second primer set. The final PCR products were analyzed using 2% agarose gel.

To study the expression of leukocyte-type 12-LOX in RV-ECT, the following pairs of primers were designed based on the leukocyte-type 12-LOX sequence characterized from rat brain and used for nested PCR: first-round PCR primers, GCCCAGGAGCCAAACGACAT (lower primer) and CATCTTCTGAGGGGACACTT (upper primer). The expected size of the first-round PCR product was 695 bp. The expected size of second-round PCR product was 342 bp using GCATTAGGAACCCAGTAGAA (lower primer) and ACCTATTGCTCATTTGTGTCC (upper primer) as second-round PCR primers.

### Immunoblot analysis of 12-LOX expression

Semiconfluent confluent (70% to 80%) HUVEC, HMVEC, RV-EC, RV-ECT, and CD4 endothelial cells were rinsed with ice-cold PBS, scraped into lysis buffer containing 20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L leupeptin, 0.15 mmol/L pepstatin A, 1 mmol/L dithiothreitol, and 1% NP-40. Protein concentration was measured using BCA protein assay kit (Pierce, Rockford, IL). Human platelet lysates (10–40 ng) or human epidermoid carcinoma A431 cell lysates (30  $\mu\text{g}$ ) were used for positive control. Cell lysates (80  $\mu\text{g}$ ) from each sample were loaded onto a minigel for electrophoresis separation. The proteins in the gel were then transferred onto a polyvinylidene difluoride membrane and processed for immunodetection using a rabbit polyclonal antibody to human platelet-type 12-LOX obtained from Oxford Biomedical Research (Oxford, MI). This antibody reacts strongly with platelet-type 12-LOX from various species, with slight cross-reactivity with 5-LOX and 15-LOX at higher concentrations. Horse-radish peroxidase-conjugated goat antirabbit IgG antibodies and enhanced chemiluminescent reagent was purchased from Amersham (Arlington Heights, IL).



**Figure 1. Scheme of AA metabolism.** The AA released from phospholipids by PLA2 activity is metabolized by the COX pathways to various prostaglandins and thromboxanes (left), by the LOX pathways to various HETEs, leukotrienes, and lipoxins<sup>42-44</sup> (middle), and by P-450 epoxygenase to epoxyeicosatrienoic acids (right). The proposed site of action for the inhibitors used in this study is shown in parentheses.

### Measurement of 12(S)-HETE levels by enzyme immunoassay

To measure 12(S)-HETE synthesis in cell culture, RV-ECT cells ( $4 \times 10^6$  cells) were plated and grown to 80% to 90% confluence in DMEM supplemented with 10% FBS and then serum starved overnight in serum-free DMEM. Fresh serum-free DMEM with 1  $\mu\text{mol/L}$  arachidonic acid was added 1 hour before VEGF treatment. BHPP was added to a final concentration of 10  $\mu\text{mol/L}$  30 minutes before VEGF treatment. Recombinant human VEGF was added in a final concentration of 10 ng/mL. After 30 minutes of treatment, cells were washed in PBS once and harvested using cell scrapers. After centrifugation, the cell pellets were resuspended in cell lysis buffer and sonicated. Lipids were extracted from cell lysates by adding ethanol to a final concentration of 15%. After centrifugation at 375g for 10 minutes at 4°C, the supernatants were acidified to pH 3.5 with 3% formic acid and applied to BAKERBOND spe Octadecyl (C18) columns (J.T. Baker, Phillipsburg, NJ). After washing with ddH<sub>2</sub>O, 15% ethanol, and petroleum ether, lipids were eluted with ethyl acetate and dried under N<sub>2</sub> gas, and 12(S)-HETE levels were measured using an EIA kit according to the manufacturer's instructions (Assay Designs, Ann Arbor, MI). To measure 12(S)-HETE levels in Matrigel (Becton Dickinson, Bedford, MA) implants, resected Matrigel plugs were homogenized in cell lysis buffer and processed for lipid extraction and 12(S)-HETE measurement.

### Stable transfection of CD4 endothelial cells and characterization

Semiconfluent CD4 endothelial cells were transfected with a pcDNA 3.1 expression construct containing human platelet-type 12-lipoxygenase cDNA, which was a gift from Dr Colin Funk (University of Pennsylvania). Empty vector was used as a control. Transfection was performed using lipofectin reagent (Life Technologies). Transfectants were selected using 300  $\mu\text{g/mL}$  geneticin (G418) in DMEM with 10% FBS. The expression of 12-LOX in CD4 transfectants was characterized by reverse transcription (RT)-PCR and Northern blot analysis for mRNA expression and by immunoblot for 12-LOX protein expression.

### Endothelial cell proliferation assay

HUVEC cells were used to study the effects of 12-LOX inhibitors on endothelial cell proliferation. Cells were harvested by trypsinization, resuspended in EGM-2, and plated in a 96-well plate at 2000 cells per well. After overnight incubation, the media were changed to fresh EBM-2 with 1% FBS and treated with recombinant human VEGF-A or bFGF (R&D Systems, Minneapolis, MN) plus various amounts of BHPP. The concentrations of BHPP used were 0, 1, 10, and 50  $\mu\text{mol/L}$  unless otherwise indicated. The final concentration of recombinant human VEGF and bFGF was 10 ng/mL. After 2 days, the plates were processed to quantitate the number of cells using an MTS cell proliferation assay kit (Promega, Madison, WI). The absorbance at 490 nm ( $A_{490}$ ) indicated the relative number of cells.

The determination of the *in vitro* growth kinetics of CD4 12-LOX transfectants in culture was conducted essentially as previously described.<sup>12</sup> Basically,  $2 \times 10^3$  cells were seeded onto 96-well culture plates in complete medium, and the number of viable cells at 48-hour intervals was assessed using an MTS cell proliferation assay kit. The  $A_{490}$  reading 2 to 3 hours after plating was used as a baseline. The number of cells was expressed as the percentage of increase from the  $A_{490}$  baseline.

### Endothelial cell migration assay

RV-ECT endothelial cell migration assay was performed essentially as previously described.<sup>12</sup> VEGF (10 ng/mL), bFGF (10 ng/mL), or various other treatments were placed in the lower chamber. For each treatment, at least 3 chambers were used unless otherwise indicated. The migration assay for CD4 12-LOX transfectants was conducted in a similar way except that the number of cells seeded was  $1 \times 10^5$  per chamber. The migrated cells were counted by a person unaware of the treatment regimen (blinded approach).

### Endothelial cell tube/cord formation assay

Cord-forming RV-ECT cells or CD4 transfectants ( $1 \times 10^5$ ) were plated on a 24-well plate precoated with a thin layer of Matrigel (Becton Dickinson). After overnight incubation, BHPP was added. After 24 hours of treatment, the media were removed and the confluent monolayer was overlaid with 0.5 mL diluted Matrigel (Becton Dickinson; final concentration, 5 mg/mL). After solidifying at 37°C, 0.5 mL DMEM-10% FBS medium was carefully added without disturbing the gel. The formation of a tubelike structure was monitored microscopically every 6 hours and recorded.

### Matrigel implantation assay for angiogenesis

The Matrigel (Becton Dickinson) implantation assay was performed as described by Ito et al<sup>28</sup> with the following modifications. Matrigel 0.4 mL premixed with bFGF (5  $\mu\text{g/mL}$ ) with and without BHPP (0.9 mg/mL) was injected subcutaneously into nude mice (4 mice/group). Mice were killed 5 days after injection and dissected to expose the implants for recording using an SP SZ-4060 stereomicroscope (Olympus America, Melville, NY). The amount of blood retained in the Matrigel was further assessed by measuring the hemoglobin levels using Drabkin's reagent (Sigma Diagnostics, St. Louis, MO).

## Results

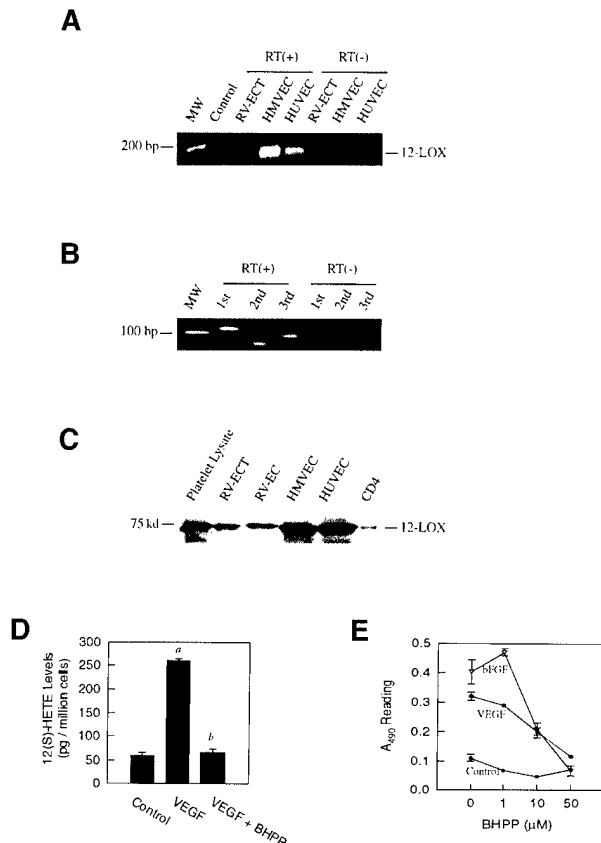
### 12-Lipoxygenase expression and activity in endothelial cells

Several studies have provided evidence that endothelial cells synthesize various lipoxygenase products such as 5(S)-HETE, 12(S)-HETE, and 15(S)-HETE.<sup>16</sup> The expression of platelet-type 12-LOX in HUVEC cells was previously detected by RT-PCR.<sup>6</sup> Using primers selective for platelet-type 12-LOX, the expression of 12-LOX mRNA was confirmed in HUVEC cells and also detected in microvascular endothelial cells, HMVEC (Figure 2A). In RV-ECT, an endothelial cell line derived from rat brain resistance microvessel, a faint band of PCR product was also present (Figure 2A). To further confirm the expression of platelet-type 12-LOX in RV-ECT cells, we designed 7 primers on the basis of the partial sequence obtained from rat Walker 256 cells.<sup>24</sup> The expression of platelet-type 12-LOX was detected by nested PCR using 3 different combinations of these 7 primers (Figure 2B). Interestingly, in addition to platelet-type 12-LOX, RV-ECT cells also expressed another isoform of 12-LOX that presumably was leukocyte-type as detected by using primers designed on the basis of the rat leukocyte-type 12-LOX sequence<sup>29</sup> (data not shown), suggesting that RV-ECT cells expressed both platelet- and leukocyte-type 12-LOX.

Immunoblot analysis using a rabbit polyclonal antibody against human platelet-type 12-LOX revealed that 12-LOX is also expressed in endothelial cells at the protein level. As shown in Figure 2C, primary cultures of HUVEC and HMVEC had the highest levels of 12-LOX expression, whereas CD4, an endothelial cell line derived from mouse pulmonary microvasculature,<sup>27</sup> had the lowest 12-LOX expression.

When RV-ECT cells were treated with VEGF for 30 minutes, a 5-fold increase in 12(S)-HETE biosynthesis was observed (Figure 2D). The increased 12-LOX activity was inhibited by pretreating RV-ECT cells with BHPP (10  $\mu\text{mol/L}$ ). Because BHPP is 20-fold more selective toward platelet-type 12-LOX than leukocyte-type 12-LOX, the results suggest that the increased 12(S)-HETE synthesis on VEGF treatment was probably caused by the increased activity of platelet-type 12-LOX.

It has been shown that arachidonic acid metabolism through the LOX pathways, especially the 12-LOX pathway, is involved in endothelial cell proliferation stimulated by serum or bFGF.<sup>13,14</sup> As



**Figure 2. Expression of platelet-type 12-LOX in endothelial cells and its role in cell proliferation.** (A) RT-PCR detection of 12-LOX expression in endothelial cells. Total RNA was isolated and processed for double-round RT-PCR using primers designed on the basis of human platelet-type 12-LOX sequence as described in "Materials and Methods." Control, no RNA present in PCR or RT reaction mixtures as controls for the quality of PCR; RT(-), no reverse transcriptase present in RT reaction mixtures as controls for the possible contamination of DNA in RNA samples; RT(+), reverse transcription present. (B) RT-PCR detection of platelet-type 12-LOX expression in RV-ECT cells. The primer combinations for 1st, 2nd, and 3rd are described in "Materials and Methods." The target sizes of the final PCR product from 1st, 2nd, and 3rd primer combinations are 111 bp, 62 bp, and 88 bp, respectively. (C) Immunoblot analysis of 12-LOX expression in endothelial cells. The blot was probed with a rabbit polyclonal antibody to human platelet-type 12-LOX. (D) Inhibition of VEGF-stimulated 12-LOX activity by BHPP. Cell treatment and measurement of 12(S)-HETE are detailed in "Materials and Methods." Columns, average levels of 12(S)-HETE per  $1 \times 10^6$  cells ( $n = 3$ ); bars, SE. a,  $P < .01$  when compared with the unstimulated control; b,  $P < .05$  when compared to the VEGF-stimulated cells. (E) Involvement of endothelial 12-LOX in bFGF- or VEGF-stimulated cell proliferation. HUVEC cells were plated in 96-well culture plates. Cell proliferation was stimulated with 10 ng/mL bFGF (open triangle) or 10 ng/mL VEGF (filled circle) in EBM-2 with 2% FBS. Cells with no bFGF or VEGF stimulation were used as controls (open circle). 48 hours after treatment with graded levels of BHPP, cell numbers were measured by an MTS method as described in "Materials and Methods." Data point, mean from quadruplicate determination; bars, SE from quadruplicate of treatment.

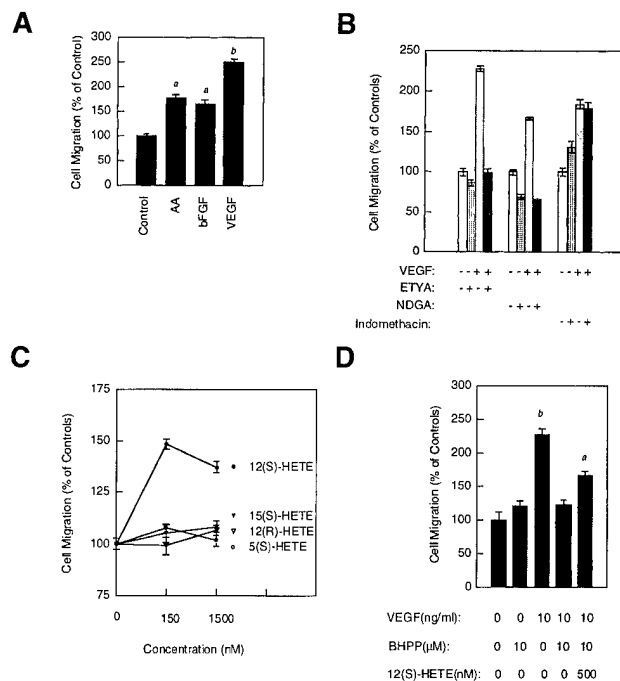
shown in Figure 2E, inhibition of 12-LOX activity by BHPP significantly inhibited bFGF- or VEGF-stimulated HUVEC proliferation, suggesting that 12-LOX activity is required for the endothelial cell proliferative responses to bFGF or VEGF.

#### Involvement of endogenous 12-lipoxygenase in endothelial cell migration

Endothelial cell migration is a requisite step in angiogenesis. It has been shown that the activation of phospholipase  $A_2$  is required for endothelial cell migration in response to bFGF.<sup>30</sup> To study whether AA released by phospholipase  $A_2$  modulates endothelial cell migration, we selected the RV-ECT cell line, which can grow in DMEM-10% FBS without bFGF or VEGF supplementation,<sup>26</sup> for

cell migration assay. First we examined the migratory response of RV-ECT cells toward exogenous AA, bFGF, and VEGF. As shown in Figure 3A, AA increased endothelial cell migration by 70% to 80%, a level comparable to that of bFGF but less than VEGF. This observation is consistent with the report that VEGF is a stronger chemotactic factor than bFGF.<sup>31</sup> Because mobilization of AA has been observed in endothelial cells on stimulation with angiogenic factors such as VEGF,<sup>32</sup> bFGF,<sup>33</sup> and angiogenin,<sup>34</sup> we studied the effect of various inhibitors of arachidonic acid metabolism on endothelial cell migration stimulated by VEGF. As shown in Figure 3B, ETYA, a promiscuous inhibitor for AA metabolism, inhibited VEGF-stimulated RV-ECT migration. NDGA, a general LOX inhibitor, also significantly reduced VEGF-stimulated RV-ECT migration. In contrast, indomethacin, a general COX inhibitor, had no effect. The results suggest that the LOX pathway, but not the COX pathway, of AA metabolism is involved in RV-ECT migration.

In the LOX pathways, AA can be used by 5-LOX to synthesize 5(S)-HETE and leukotrienes, by 12-LOX to synthesize mainly 12(S)-HETE, and by 15-LOX to synthesize 15(S)-HETE. To delineate which pathway(s) is involved in endothelial cell migration, we first studied the effect of various types of HETEs on RV-ECT cell migration. As shown in Figure 3C, among the various types of HETEs tested, only 12(S)-HETE, but not 5(S)-HETE, 15(S)-HETE, or 12(R)-HETE, could stimulate endothelial cell migration. The stimulation of endothelial cell migration by 12(S)-HETE is consistent with previous observations.<sup>12</sup> To study whether the endogenous synthesis of 12(S)-HETE is involved in endothelial cell migration in response to VEGF, we examined the effect of BHPP on VEGF-stimulated endothelial cell migration. As shown in



**Figure 3. Arachidonate metabolites in endothelial cell migration.** The migration assays were performed as described in "Materials and Methods." (A) Stimulation of RV-ECT migration by arachidonic acid (1 μmol/L), bFGF (10 ng/ml), and VEGF (10 ng/ml). (B) VEGF-stimulated RV-ECT migration involves lipoxygenase-dependent arachidonic acid metabolism. Treatment: VEGF, 10 ng/mL; ETYA, 5 μmol/L; NDGA, 50 μmol/L; indomethacin, 50 μmol/L. -, absence of treatment (vehicle only); +, presence of treatment. (C) Differential effects on RV-ECT cell migration by various HETEs. (D) Modulation of RV-ECT cell migration by 12-LOX inhibitor BHPP and 12(S)-HETE. Columns, percentage of the average number of cells migrated when compared with the controls; bars, SE (a,  $P < .05$ ; b,  $P < .01$ ; Student's *t* test). All migration assays were repeated at least 4 times.

Figure 3D, BHPP inhibited VEGF-stimulated RV-ECT endothelial cell migration. Furthermore, exogenous 12(S)-HETE partially reversed the inhibitory effect of BHPP on RV-ECT migration (Figure 3D). We also tested the effect of an inhibitor of the 5-LOX pathway, ie, MK886, on VEGF-stimulated RV-ECT migration and did not observe any appreciable effects at a concentration of 10  $\mu\text{mol/L}$  (data not shown). These results collectively suggest that 12-LOX activity and 12(S)-HETE are involved in endothelial cell migration.

#### Endothelial 12-lipoxygenase was involved in RV-ECT tube differentiation

Certain endothelial cell lines, under proper culture conditions, have the capacity to form tubelike structures. Confluent RV-ECT cells can spontaneously form cordlike structures under normal culture conditions.<sup>26</sup> Therefore, this cell line was chosen for the current study. When RV-ECT cells were cultured between 2 layers of Matrigel, the formation of cordlike structures was expedited, as manifested by the formation of numerous vacuoles within 4 to 5 hours and interconnected tubelike structures within 24 hours (Figures 4A, 4C). Pretreatment of RV-ECT cells with BHPP (10  $\mu\text{mol/L}$ ) significantly impaired their ability to form cordlike structures (Figures 4B, 4D), suggesting the potential involvement of 12-LOX in endothelial cell cordlike differentiation.

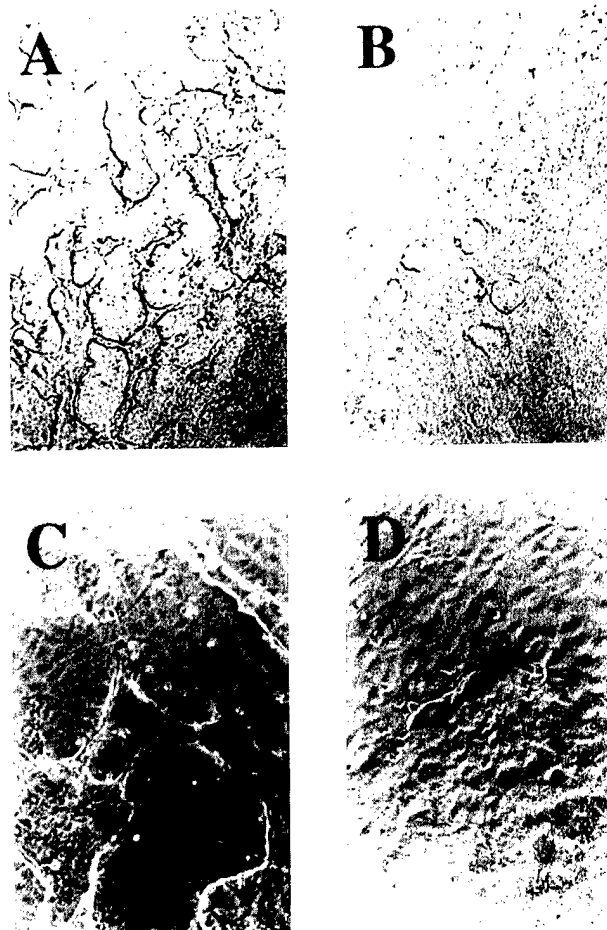
#### Endothelial cells that overexpress 12-LOX had increased motility and enhanced tubelike differentiation

To further explore the role of 12-LOX in endothelial cell migration and tubelike differentiation, we transfected CD4 endothelial cells with a pcDNA-12-LOX expression construct. The CD4 endothelial cell line was selected for 12-LOX overexpression study because of its low level of 12-LOX expression (Figure 2C). Stable transfectants were selected using G418, and the transfectant pools were characterized for the expression of 12-LOX mRNA by RT-PCR (Figure 5A) and Northern blot analysis (Figure 5B). The expression of 12-LOX was also increased in 12-LOX-transfected CD4 cells at protein levels as revealed by Western blot (Figure 5C). The growth rate of 12-LOX-transfected CD4 endothelial cells was similar to the mock transfectants (data not shown), suggesting that although 12-LOX is involved in bFGF- or VEGF-stimulated endothelial cell growth, the overexpression of 12-LOX in CD4 cells is not sufficient to stimulate endothelial cell proliferation. However, the overexpression of 12-LOX was able to stimulate CD4 endothelial cell migration (Figure 5D). Further, the increased motility in 12-LOX-transfected CD4 cells was inhibited by BHPP, suggesting that it is the increased 12-LOX activity in endothelial cells that enhances cell motility (Figure 5D).

When cultured within 2 layers of Matrigel, CD4 vector transfectants did not form tubelike structures (Figure 5E, left panel). In contrast, under identical conditions, CD4 12-LOX transfectants retracted and formed tubelike structures (Figure 5E, right panel). The results further suggest the involvement of 12-LOX in endothelial cell tube differentiation.

#### Inhibition of angiogenesis in vivo by 12-lipoxygenase inhibitor

The involvement of the 12-LOX pathway of arachidonic acid metabolism in endothelial cell proliferation, migration, and tube differentiation led us to study whether the inhibition of 12-LOX activity can compromise angiogenesis in vivo. Because the induction of angiogenesis in vivo by bFGF requires the angiogenic activities of VEGF,<sup>35</sup> we used bFGF premixed with Matrigel to induce angiogenesis. As shown in Figure 6A, bFGF induced

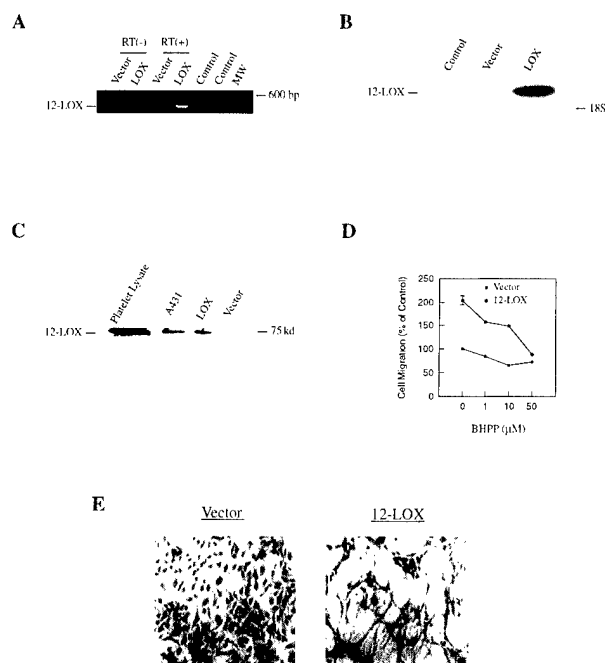


**Figure 4. Involvement of 12-LOX in endothelial cell tubelike differentiation.** The formation of cordlike structures by RV-ECT cells was facilitated by Matrigel as detailed in "Materials and methods." (A) RV-ECT treated with ethanol as control. Original magnification,  $\times 40$ . (B) RV-ECT treated with 10  $\mu\text{mol/L}$  BHPP. Original magnification,  $\times 40$ . (C) RV-ECT treated with ethanol as control. Original magnification,  $\times 100$ . (D) RV-ECT treated with 10  $\mu\text{mol/L}$  BHPP. Original magnification,  $\times 100$ . Shown here are typical observations from 4 independent studies on the tube-forming ability of RV-ECT and the effect of BHPP.

massive angiogenesis around and within the implant (upper panel, left). When dissected out, the implanted Matrigel retained a large volume of blood within the gel (upper panel, right). Matrigel implants without bFGF had little or no angiogenic activities in vivo (bottom panel). Inclusion of the 12-LOX inhibitor BHPP in the implants significantly reduced the ability of bFGF to induce angiogenesis (Figure 6A, middle panel), suggesting that 12-LOX is involved in angiogenesis in vivo. Figure 6B shows the hemoglobin levels in the dissected Matrigel. As shown in the Figure, BHPP significantly reduced the hemoglobin levels in Matrigel ( $P < .05$ ), suggesting a reduction of angiogenesis. The reduction of angiogenesis was closely correlated with the levels of 12(S)-HETE in the Matrigel implants as shown in Figure 6C. Taken together, the data suggest a critical role of 12-LOX activity in angiogenesis in vivo.

## Discussion

In this study we demonstrated that endothelial cells from different species (rat and human) and different organs (brain, umbilical cord, and foreskin) express platelet-type 12-LOX and elucidated its important role in endothelial cell responses to angiogenic stimuli.



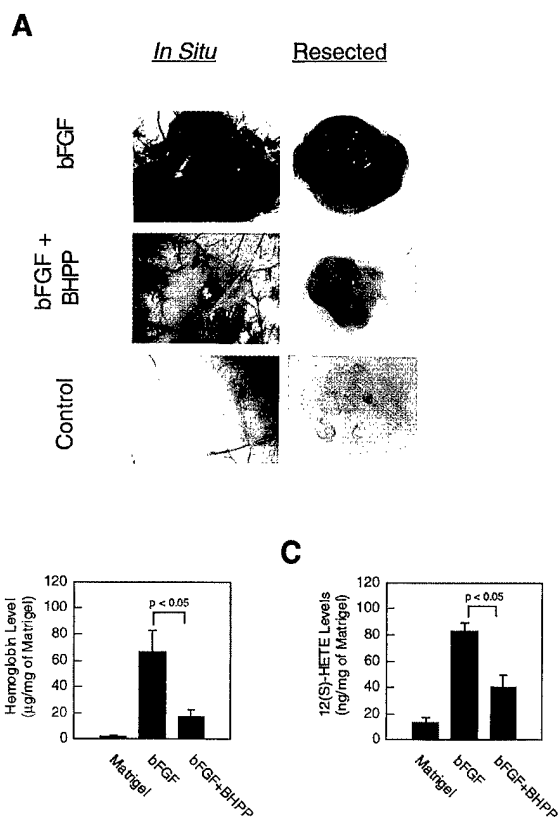
**Figure 5. Stimulation of endothelial cell migration and tubelike differentiation by the overexpression of 12-LOX in endothelial cells.** CD4 cells were transfected with a 12-LOX expression construct or an empty vector as a control. (A) Detection of 12-LOX mRNA expression by RT-PCR. Primers were the pair of the primers for the first round of PCR. The reaction was carried out for 30 cycles. Control, no RNA present in PCR or RT reaction mixtures as controls for the quality of PCR; RT(-), no reverse transcription present in RT reaction mixtures as controls for the possible contamination of DNA in RNA samples; RT(+), reverse transcription present. Vector, CD4 cells transfected with pcDNA 3.1; LOX, CD4 cells transfected with pcDNA construct with 12-LOX cDNA insert. (B) Northern blot analysis of 12-LOX mRNA levels. Control, loading buffer as the blank control. (C) Analysis of 12-LOX expression at the protein level by immunoblot. (D) Increased cell migration in CD4 12-LOX transfectants. The migration assay was performed as detailed in "Materials and methods" using CD4 cells transfected with pcDNA (open circle) or pcDNA 12-LOX construct (filled circle). Data point, mean from 30 fields counted; bars, SE. The migration assay was repeated for 3 times with similar results. (E) Increased formation of tubelike structures in CD4 12-LOX transfectants. Left panel, vector control; right panel, 12-LOX transfected CD4 cells.

Inhibition of 12-LOX activity by BHPP, a platelet-type selective inhibitor, attenuated the endothelial cell mitogenic and the migratory responses to the angiogenic factors bFGF and VEGF and the tubelike differentiation on Matrigel. Forced expression of 12-LOX in the CD4 endothelial cells stimulated cell migration and promoted tube differentiation. Inhibition of 12-LOX activity by BHPP significantly reduced angiogenesis *in vivo*. Our findings suggest that eicosanoids from the arachidonic acid metabolism through the 12-LOX pathway, ie 12(S)-HETE, are involved in modulating angiogenesis.

There are seemingly conflicting reports regarding the isozymes of 12-LOX expressed in endothelial cells as both leukocyte type 12-LOX<sup>4</sup> and platelet-type 12-LOX<sup>6</sup> are reported. In the current study, we demonstrated that platelet-type 12-LOX was expressed in HUVEC and HMVEC as well as in RV-ECT, an endothelial cell line originally isolated from rat brain resistance blood vessels.<sup>26</sup> RV-ECT cells also express another isoform of 12-LOX originally isolated from rat brain and close to leukocyte-type.<sup>29</sup> When RV-ECT cells were treated with VEGF, there was a 5-fold increase in 12(S)-HETE biosynthetic activity inhibitable by BHPP pretreatment. Because BHPP is much more selective toward platelet-type 12-LOX than leukocyte-type (Furstenberger G, personal communication), the results suggest that it is the platelet-type 12-LOX, not

the leukocyte-type 12-LOX, that is activated in endothelial cells during angiogenic responses.

Arachidonic metabolites have been implicated in angiogenesis since the inhibition of arachidonic acid metabolism by  $\alpha$ -guaiaconic acid (GR-12) attenuated endothelial cell migration, tube formation, and angiogenesis *in vivo*.<sup>36</sup> Cellular mobilization of AA is usually achieved by PLA<sub>2</sub> cleavage of phospholipids. The activation of PLA<sub>2</sub> and the subsequent mobilization of AA have been observed in endothelial cells in response to various extracellular cues such as angiogenin, bFGF, zinc, and phorbol ester.<sup>33,34,37,38</sup> As a potent angiogenic factor, bFGF stimulates the migration and proliferation of vascular endothelial cells. Abrogation of the release of AA in endothelial cells by the inhibition of PLA<sub>2</sub> activity inhibited bFGF-stimulated cell proliferation<sup>14</sup> and migration.<sup>30</sup> VEGF also can rapidly increase phosphorylation and activity of cytosolic PLA<sub>2</sub> and stimulate the release of AA in HUVEC.<sup>32</sup> In a recent study, it was shown that the inhibition of PLA<sub>2</sub> activity in granuloma by SB 203 347 significantly reduced angiogenesis,<sup>39</sup> suggesting that the activation of PLA<sub>2</sub> and the subsequent mobilization of AA and lysophospholipid are intrinsic steps during angiogenesis.



**Figure 6. Attenuation of angiogenesis in vivo by 12-LOX inhibitors.** Matrigel implantation assay for angiogenesis was performed as described in "Materials and methods" and repeated at least 3 times with similar results. (A) Matrigel implantation angiogenesis assay. Top panel: left, bFGF (5  $\mu$ g/mL Matrigel) in situ; right, bFGF (5  $\mu$ g/mL Matrigel) resected Matrigel. Middle panel: left, bFGF (5  $\mu$ g/mL Matrigel) and BHPH (0.8 mg/mL Matrigel) in situ; right, bFGF (5  $\mu$ g/mL Matrigel) and BHPH (0.8 mg/mL Matrigel) resected implant. Bottom panel: left, Matrigel alone in situ; right, resected Matrigel blank control. (B) Hemoglobin levels in resected implants. The hemoglobin was measured by Drabkin's reagent. Columns, hemoglobin levels normalized with protein concentrations. Bars, SE from quadruplicate samples. (C) 12(S)-HETE levels in resected implants. Lipids were extracted from the resected implants, and 12(S)-HETE levels were measured as described in "Materials and methods." Columns, average 12(S)-HETE levels normalized with protein concentrations. Bars, SE ( $n = 4$  for Matrigel control;  $n = 3$  for bFGF;  $n = 5$  for bFGF plus BHPH).

The downstream events for released AA include the synthesis of various prostaglandins and thromboxanes through the COX pathway, various HETEs and lipoxins<sup>42-44</sup> through the LOX pathways, and various epoxyeicosatrienoic acids through cytochrome P-450 epoxygenase. In this study, we found that ETYA, a general inhibitor for arachidonic acid-derived metabolism, and NDGA, an agent that can inhibit LOX activity, inhibited endothelial cell migration stimulated by VEGF. On the other hand a general COX inhibitor, indomethacin, had no appreciable effect. In contrast, BHPP, which is a selective platelet-type 12-LOX inhibitor, blocked VEGF-stimulated endothelial cell migration. The involvement of 12-LOX and its AA metabolite in endothelial cell migration was further strengthened by the observations that exogenously added 12(S)-HETE directly stimulated RV-ECT migration and partially reversed the inhibitory effect of BHPP on cell migration. Finally, the overexpression of 12-LOX in CD4 endothelial cells significantly stimulated cell migration in a 12-LOX activity-dependent manner. The findings collectively suggest the role of endothelial 12-LOX and its AA metabolite, 12(S)-HETE, in endothelial cell migration.

In addition to its role in endothelial cell migration, we found that 12-LOX is involved in endothelial cell tube formation. Pretreatment of the RV-ECT cell line with BHPP significantly inhibited the formation of vessel-like structures within Matrigel. The second line of evidence is the observation that the overexpression of 12-LOX in CD4 endothelial cells promoted the formation of tubelike structures on Matrigel. Interestingly, it has been extensively documented that exogenous 12(S)-HETE can induce a reversible retraction of endothelial cell monolayers cultured on collagen by regulating PKC and  $\alpha_v\beta_3$  integrin.<sup>40,41</sup> It remains to be determined, however, whether endothelial cell retraction is an early event of tube differentiation on matrix proteins such as collagens or Matrigel. Studies are under way to determine this potential relationship.

It has been reported that the 12-LOX pathway of AA metabolism is required in bFGF-stimulated endothelial cell proliferation.<sup>14</sup> We demonstrated that the inhibition of 12-LOX activity also compromised VEGF-stimulated endothelial cell proliferation. The role of 12-LOX in endothelial cell proliferation, together with the finding that 12-LOX is involved in endothelial cell migration and tube differentiation, implicate the mobilization of arachidonic acid and the generation of 12(S)-HETE through the 12-LOX pathway in endothelial cells as an early event in the intracellular cascade of angiogenic responses. Currently we are exploring the mechanism

by which 12-LOX and 12(S)-HETE participate in the signaling events elicited by VEGF or bFGF in endothelial cells.

The involvement of 12-LOX in endothelial cell angiogenic responses *in vitro* is further corroborated by the observation that the 12-LOX inhibitor BHPP significantly reduced bFGF-stimulated angiogenesis *in vivo*. Because bFGF induces angiogenesis by modulating endothelial cell expression of VEGF, which in turn contributes to angiogenesis in an autocrine mechanism,<sup>35</sup> BHPP may have inhibited angiogenesis by attenuating endothelial cell angiogenic responses to bFGF and VEGF.

It should be noted that although platelet-type 12-LOX uses arachidonic acid to synthesize 12(S)-HETE almost exclusively, platelet-type 12-LOX has been shown to use leukotriene A<sub>4</sub> to synthesize lipoxin<sup>42-44</sup> and also 5(S)-HETE and 15(S)-HETE to form 5(S), 12(S)-DiHETE and 14(R), 15(S)-DiHETE, respectively.<sup>45</sup> It awaits further studies regarding whether other 12-LOX products, in addition to 12(S)-HETE, is angiogenic. *In vivo*, 12(S)-HETE is a prominent product of arachidonic acid metabolism through the LOX pathway in platelets. The pro-angiogenic function of 12(S)-HETE as delineated in the current study and in our previous reports<sup>12,13</sup> implicates the possible involvement of platelets in angiogenesis during tumor growth and metastasis. Indeed, platelets are intimately involved in tumor angiogenesis,<sup>46,47</sup> and platelet aggregation stimulates the release of VEGF.<sup>48</sup> Clinically, 30% to 60% of patients with advanced cancer have platelet abnormalities such as thrombocytosis and many other thromboembolic disorders. In addition, activated platelets have been frequently associated with many malignant tumors.<sup>49</sup> Obviously, the involvement of platelets adds to the complexity of tumor angiogenesis regulation.

In summary, our data suggest the important role of 12(S)-HETE generated by the 12-LOX pathway in angiogenesis and suggest the possibility of using 12-LOX inhibitors to treat angiogenic diseases such as tumor growth and arthritis. Studies are under way to evaluate the efficacy of 12-LOX inhibitors against solid tumor growth *in vivo*.

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## **Identification of 12-Lipoxygenase Interaction with Cellular Proteins by Yeast Two-Hybrid Screening**

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### Identification of 12-Lipoxygenase Interaction with Cellular Proteins by Yeast Two-Hybrid Screening<sup>†</sup>

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**ABSTRACT:** The platelet isoform of 12-lipoxygenase (12-LOX) is expressed in a variety of human tumors. 12-LOX metabolizes arachidonic acid to 12(*S*)-hydroxyeicosatetraenoic acid (12(*S*)-HETE), which induces a number of cellular responses associated with tumor progression and metastasis. Little is known about 12-LOX regulation and no direct regulators of 12-LOX activity have been identified. To identify potential regulators of 12-LOX, we isolated cDNAs encoding 12-LOX interacting proteins using the yeast two-hybrid system. We screened a yeast two-hybrid interaction library from human epidermoid carcinoma A431 cells and identified four cellular proteins that interact specifically with 12-LOX. We identified type II keratin 5, lamin A, the cytoplasmic domain of integrin  $\beta 4$  subunit and a phosphoprotein C8FW as 12-LOX interacting proteins. Here, we demonstrated that keratin 5, a 58 kD protein required for formation of 8 nm intermediate filaments, binds to 12-LOX in human tumor cells and may contribute to the regulated trafficking of 12-LOX. We also showed that lamin A binds 12-LOX in human tumor cells. These proteins provide the first candidate regulators of 12-LOX.

12-lipoxygenase (EC 1.13.11.31), one of at least three lipoxygenases, metabolizes arachidonic acid (AA) to 12-hydroperoxyeicosatetraenoic acid (12-HPETE),<sup>1</sup> which is subsequently converted to 12(*S*)-HETE and hepoxilins (*I*). Enzymological, immunological, and molecular biological evidence indicates that the 12-LOX proteins expressed in

leukocytes, platelets, and tracheal epithelium are three distinctly different enzymes (2–3). Platelet-type 12-LOX is expressed normally in platelets, megakaryocytes, umbilical vein endothelial cells, and a wide variety of human and rodent tumor cells including HEL (human erythroleukemia) cells and A431 cells (human epidermoid carcinoma) (4). Platelet 12-lipoxygenase is a dual-function enzyme that possesses both oxygenase and lipoxin synthase activity (5). Platelet 12-LOX metabolizes only AA (but not *c*-18 fatty acids, such as linoleic acid) to form exclusively 12(*S*)-HETE (6) and utilizes leukotriene A<sub>4</sub> to generate lipoxin A<sub>4</sub> and B<sub>4</sub> during platelet–leukocyte interactions (5). 12(*S*)-HETE induces a plethora of responses in tumor cells and is linked to tumor progression and metastasis (7). For example, 12(*S*)-HETE has been reported to stimulate integrin expression and secretion of proteinases, enhance tumor cell motility and invasion, and induce angiogenesis (7–8). Leukotrienes and

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<sup>1</sup> Abbreviations: A431 cell, human epidermoid carcinoma; 12-LOX, 12-lipoxygenase; 12(*S*)-HETE, 12(*S*)-hydroxyeicosatetraenoic acid; AA, arachidonic acid; HEL, human erythroleukemia; Cdk, cyclin-dependent kinase; FLAP, 5-lipoxygenase activating protein.

lipoxins participate in multicellular events including thrombosis, inflammation, immunity, and atherosclerosis. For instance, lipoxin A<sub>4</sub> down-regulates leukocyte responses and trafficking and neutrophil-endothelial interaction (9–10), while lipoxin B<sub>4</sub> regulates human neutrophil adherence and motility (11).

Human platelet-type 12-LOX is believed to play a role in cancer and other pathological conditions, such as psoriasis, atherosclerosis, and arthritis. In a clinical study of 137 prostate cancer patients, platelet-type 12-LOX expression levels were determined in cancer tissue and compared with the levels in matching normal tissue from each patient. Approximately 38% of the patients studied exhibited elevated levels of platelet-type 12-LOX in the cancer tissues. This elevated 12-LOX correlated positively with tumor stage and grade and positivity for prostate cancer cells in the surgical margins (12). In addition 12-LOX activity is up regulated in tumor cells following  $\gamma$  radiation (13). Clearly, understanding how the 12-LOX enzyme is regulated will be an important step in elucidating the role of this enzyme in a variety of human cancers.

The enzyme activity of 12-LOX and 5-LOX is regulated by a translocation process following cell stimulation. It has been shown that Ca<sup>2+</sup> and thrombin increase 12-LOX activity and 12(S)-HETE production by the translocation of 12-LOX from cytosol to membrane in human platelets, HEL cells, and A431 cells (14–15, 33, 41). A recent report demonstrates that an anti-platelet agent inhibits the 12-LOX activity and 12(S)-HETE production by blocking the translocation of 12-LOX (15). However, to date there is no direct evidence for protein(s) that could mediate 12-LOX translocation, and no direct regulator/interacting protein of 12-LOX has been found. In contrast, for leukocyte 5-LOX, there is evidence of complex protein-protein interactions in its nuclear membrane translocation, activation, and substrate acquisition in intact cells (27). For example, FLAP (5-lipoxygenase-activating protein) association with 5-LOX after its translocation acts as an arachidonic acid transfer/docking protein that “presents” the substrate to 5-LOX on the leukocyte nuclear membrane (16). This finding was the first indication that a protein directly associates with and regulates the activity of a member of the lipoxygenase family. Recently, three additional 5-LOX-interacting proteins that may be involved in regulation and/or nuclear localization were identified using the yeast two-hybrid system (17).

In this study, we used a yeast two-hybrid approach to identify proteins that interact with 12-LOX and that may regulate its activity. We constructed a two-hybrid library from A431 cells and identified from it four distinct cellular proteins that interacted specifically with 12-LOX. They are human type II keratin K5, nuclear envelope protein lamin A, integrin  $\beta$ 4 cytoplasmic domain, and human C8FW phosphoprotein. The identification of these 12-LOX-interacting proteins may help us to understand the complex ways in which 12-LOX is involved in tumor progression and metastasis.

## MATERIALS AND METHODS

**Yeast Strains and Manipulation.** *Saccharomyces cerevisiae* yeast strains used were RFY231 (MATa *his3 ura3-1 trp1 $\Delta$ ::hisG leu2::3Lexop-LEU2*) (19) and RFY206 (MATa *his3 $\Delta$ 200 leu2-3 lys2 $\Delta$ 201 ura3-52 trp1 $\Delta$ ::hisG*) (21). Yeast were

grown using standard microbiological techniques and media (22–23). Media designations are as follows: YPD is YP (yeast extract plus peptone) medium with 2% glucose. Minimal dropout media are designated by the component that is left out (e.g., -ura -his -trp -leu medium lacks uracil, histidine, tryptophan, and leucine). Minimal media contained either 2% glucose (Glu) or 2% galactose plus 1% raffinose (Gal). X-Gal minimal dropout plates contained 40 mg/mL X-Gal and phosphate buffer at pH 7.0. DNA was introduced into yeast by LiOAc-mediated transformation as described (24).

**Human A431 Cell cDNA Library Construction.** RNA was isolated from human epidermoid carcinoma A431 cells, and mRNA was purified using Oligo-tex Beads mRNA kit (QIAGEN Inc, Valencia, CA). cDNA was synthesized with the Stratagene cDNA synthesis kit, essentially according to the manufacturer's instructions. Briefly, poly(A)<sup>+</sup> mRNA derived from human epidermoid carcinoma A431 cells was used to direct the synthesis of first-strand cDNA by reverse transcriptase with an XhoI-oligo d(T) primer. Following second-strand synthesis, the cDNA was ligated to an EcoRI adaptor, digested with XhoI, and subsequently size selected over cDNA Size Fractionation Columns from Gibco BRL. Fractions containing cDNAs ranging from 500 bp to > 3 kb in length were pooled and subcloned into the yeast library plasmid pJG4-5 (25). The average insert size was evaluated by purifying plasmid DNA from 48 random clones and digesting with EcoRI+XhoI. The size of each product was determined by gel electrophoresis. The A431 cDNA had  $3.0 \times 10^6$  independent clones, and 90% of the plasmids had cDNA inserts of 0.3–3.8 kb (average size, 1.2 kb). The resultant cDNA library contains  $3.0 \times 10^6$  primary recombinant clones.

**Plasmids.** Bait plasmids expressing LexA fusion proteins were derivatives of the *HIS3* 2  $\mu$ m plasmid, pEG202 (20). The full-length cDNA encoding human platelet-type 12-LOX was provided by Dr. Colin Funk (University of Pennsylvania). The entire coding region of the cDNA was amplified by the polymerase chain reaction (PCR) with a 5' *Bam*HI site introduced in the upper primer (CCGGGGATCCG-TATGGGCCGCTACCGCATC) and the lower primer corresponding to an *Xho*I site from the original cDNA downstream of the stop codon (GCCGCGAGCTCAGTCTACCA-CTGTGACAA). The PCR product was digested with *Bam*HI and *Xho*I and inserted into the *Bam*HI/*Xho*I sites of pEG202 (20) to generate the bait plasmid pLexA-12-LOX; the plasmid encodes the entire 663 amino acid human platelet 12-LOX protein. Additional bait plasmids used in the specificity test are described elsewhere (21, 25–26).

**Yeast Two-Hybrid Screen.** The yeast two-hybrid procedures were conducted as described (28–29). Strain RFY231 containing pSH18-34 and pLexA-12-LOX was transformed with A431 interaction library DNA and a total of  $6.5 \times 10^6$  independent colonies were collected. The selection for interacting clones was performed in media containing galactose and lacking leucine. 400 Leu<sup>+</sup> yeast colonies were further tested for lacZ expression by plating them on medium containing galactose and X-Gal. 40 of the Leu<sup>+</sup> positive clones demonstrated galactose-dependent activation of both reporters, suggesting interaction between the galactose-inducible cDNA protein and 12-LOX. Library plasmids from yeast colonies, expressing the putative 12-LOX-interacting proteins, were rescued by transformation of yeast plasmid

DNA into KC8 *E. coli* followed by selection on minimal medium lacking tryptophan (29). To determine which clones were unique, PCR products generated with primers flanking the cDNA insertion site in pJG4-5 (BC01 and BC01, ref 29) were digested with *AluI* and *HaeIII* and analyzed on a 2% agarose gel. The specificity of the unique interactors was tested using the interaction mating assay as described (21). Briefly, rescued library plasmids were introduced into RFY231 and the transformants were mated with various derivatives of RFY206 that each contained pSH18-34 and a bait plasmid expressing a LexA fusion. 12 library plasmids encoded proteins that interacted with the 12-LOX bait but not unrelated baits. These 12 cDNAs were sequenced and evaluated by the Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information Internet site ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

**Immunoprecipitation.** Cells were lysed in a cold lysis buffer consisting of 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP-40, 0.1% SDS. The lysate was clarified by centrifugation at 10000g for 10 min. Isolation of nuclear extracts was according to the standard protocol (30). The supernatants and soluble nuclear extracts were immunoprecipitated with 4–6  $\mu$ L (1–2  $\mu$ g) antibody against human platelet-type 12-LOX or anti-5LOX and anti-15-LOX antibodies (Oxford Biomedical Research, Oxford, MI) or the anti-keratin (Chemicon International INC., Temecula, CA) or anti-lamin A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h, followed by 40  $\mu$ L Sepharose 4B-conjugated protein G at 4 °C overnight. Immune complexes were washed three times in the lysis buffer, and the pellets were suspended in SDS sample buffer for SDS-PAGE electrophoresis. Aliquots of total cell lysate were mixed with 1 vol of SDS sample buffer (85 mM Tris-HCl, pH 6.8, containing 1.4 (w/v) SDS, 14% (v/v) glycerol, 5% (v/v) mercaptoethanol and a trace of bromophenol blue, boiled for 5 min and subjected to SDS-PAGE on 4–20% acrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, nonspecific sites were blocked with 5% (w/v) nonfat-dry milk in TTBS (0.1% Tween-20, 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.6) for 2 h at 25 °C followed by probing primary antibody. After the blot was washed three times in TTBS, the membranes were incubated for 1 h at 25 °C with horseradish peroxidase-conjugated secondary anti-IgG (dilution: 1:4500. Amersham, Arlington Heights, IL). The blot was washed again in TTBS, then developed using ECL according to the manufacture's instructions (Amersham, Arlington Heights, IL).

**Immunofluorescent Staining.** Intracellular 12-LOX and keratin were localized using a modification (49) of the general immunocytochemical methodologies described by Willingham (50).  $2 \times 10^4$  Cells were grown in 4-well Lab-Tek chamber (Nalge Nunc Intl, Naperville, IL) in 0.5 mL media to 60–80% confluence and the media changed to serum free media the night before the experiment. Cells were washed with PBS 3 $\times$  and then fixed with 3.7% formaldehyde in phosphate-buffer saline, pH 7.4 for 10 min. Fixation and subsequent steps were performed at 25 °C for intracellular labeling. After the sample was washed with PBS, the cells were blocked with 2 mg/mL BSA in PBS. All subsequent antibody and wash solution contained 0.1% saponin. Cells

were incubated with primary antibody (rabbit anti-human 12-LOX, mouse anti-human keratin) for 2 h and washed. In controls, preimmune serum (rabbit or mouse) was substituted for the primary antibody (1:100 dilution). Cells were incubated in secondary antibody (FITC-conjugated AffiniPure Goat anti-mouse IgG or Rhodamine Red-X-conjugated AffiniPure-Goat anti-Rabbit IgG) and secondary blocking reagent for 1 h. Secondary antibody was diluted in PBS-0.1% saponin (1:100 dilution) and 5% normal goat serum was added to this solution. After the resultant was washed, the coverslips were mounted upside-down on slides with SlowFade anti-fade reagent and observed with a Zeiss LSM 310 laser confocal microscope.

**Transfection.** The full-length cDNA encoding human platelet-type 12-LOX from pCMV-12-LOX (provided by Dr. Colin Funk, University of Pennsylvania) was cloned into the *EcoRI/XbaI* sites of pcDNA3.1 (Invitrogen), which uses the neomycin-resistance gene as the selectable marker. Cells grown in 6-well plates were transfected with 3–12  $\mu$ g of pcDNA-12-LOX by the FuGENE 6 Transfection Reagent (Boehringer Mannheim Co., Indianapolis, IN) following the manufacture's instructions. Neomycin-resistant cells were selected in 300  $\mu$ g/mL Geneticin (G418; LifeTechnologies, Inc., Grand Island, NY). The cells were passaged and tested by immunoprecipitation and Western Blotting assays for enhanced 12-LOX protein expression. Cells transfected with vector alone were selected and analyzed in parallel. While in culture, cells were fed with a G418-containing medium to prevent outgrowth of revertant cells. All of the cells were frozen at early passage for subsequent study.

## RESULTS AND DISCUSSION

**A431 Interaction Library.** To identify proteins that may interact with 12-lipoxygenase, we constructed a yeast two-hybrid interaction library from A431 cells. This cell line has been widely used to study 12-LOX in tumor cells, since it expresses enzymatically active platelet-type 12-LOX protein, but not the leukocyte-type isoform (33–35). In A431 cells, the predominant amount of 12-LOX protein resides in the cytosol (33, 36). In contrast, 12-LOX enzyme activity is mainly localized in the membrane fraction (33, 37). EGF (TPA,  $\text{Ca}^{2+}$ ) increases total cellular 12-LOX protein and enhances the association of 12-LOX protein with perinuclear or cytoplasmic membrane (33, 37–41). In addition, EGF stimulates 12-LOX activity and generation of 12(S)-HETE from cellular arachidonate (33, 36–37, 41). These features make A431 cells an ideal model to study the regulation of 12-LOX activity.

To construct the cDNA library from A431 cells, we isolated poly(A)<sup>+</sup> mRNA from 80% confluent cultured A431 cells, used it to synthesize unidirectional cDNA, and inserted the cDNA into the yeast two-hybrid vector pJG4-5 (see Materials and Methods). This vector allows conditional expression (induced by galactose and repressed by glucose) of cDNA-encoded proteins with a transcription activation domain at their amino termini. The resulting library had  $3.0 \times 10^6$  independent clones, of which 90% had inserts with sizes ranging from 0.3 to 3.8 kb (average size 1.2 kb).

**12-Lipoxygenase Interacting Proteins.** To identify potential regulators of 12-LOX, we screened the A431 library for clones that encoded 12-LOX-interacting proteins (see Ma-

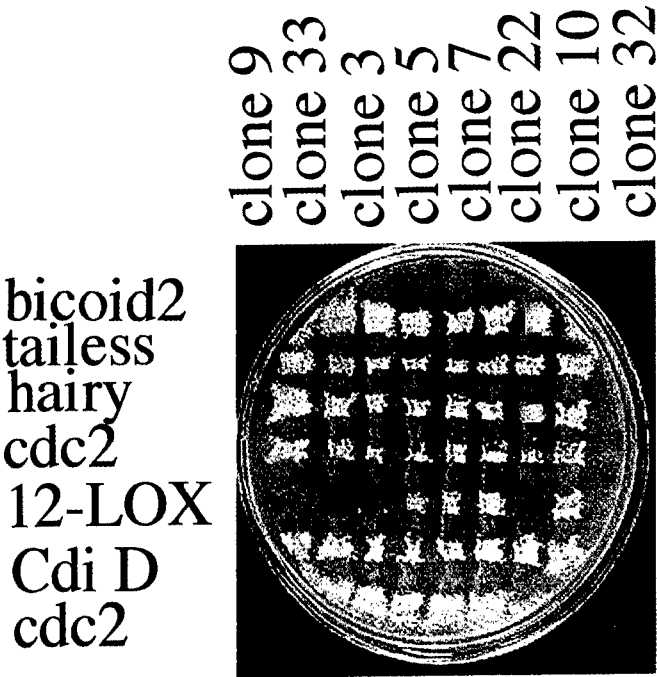


FIGURE 1: Specificity of 12-LOX interactors in the yeast two-hybrid assay. Representative plate of a mating assay to test the specificity of 12-LOX interactors. This plate demonstrates the interactions between seven bait proteins (horizontal lines of yeast) and eight prey proteins (vertical lines) on a Gal/Raf X-Gal plate (urp-his-trp- drop out). The strength of the interaction is suggested by the level of activation of lacZ reporter as indicated by blue color, as summarized in Table 1.

terials and Methods). Yeast expressing the full-length platelet isoform of 12-LOX fused to the LexA DNA-binding domain were transformed with the A431 interaction library. From  $6.5 \times 10^6$  yeast transformants, we identified 12 independent clones in which the LexA-driven reporters were active only in galactose, indicating that they contained cDNA-encoded proteins that interacted with LexA-12-LOX. We isolated the library plasmids from these clones and determined that they represented four different cDNAs. Six identical clones encoded the carboxy-terminal 330 amino acid residues of type II keratin K5. Two clones encoded the carboxy-terminal 202 residues of the nuclear envelope protein lamin A. Another two clones encoded the cytoplasmic domain of integrin  $\beta 4$ . The final two clones encoded a phosphoprotein, C8FW, with some similarity to protein kinases. To further determine the specificity of the four 12-LOX interactors, we conducted a two-hybrid mating assay to test for interactions with LexA-12-LOX and with other unrelated LexA fused proteins. As shown in Figure 1 and Table 1, all four clones interacted specifically with 12-LOX, but not with several other proteins including *Drosophila* cyclin D, Cdk1, hairy, tailless, or bicoid.

**Human Keratin K5 Interacts with 12-LOX in A431 Cells.** Keratins are major components of intermediate filaments. In epithelial cells, cytoskeletal intermediate filaments contain type I and type II keratins. The 58 kD K5 protein is essential for formation of 8-nm intermediate filaments, disruption of which may reduce tumor invasion and metastasis (42–43). For example, disruption of intermediate filaments has been shown to inhibit the expression of integrins on the surface of tumor cells (44), decrease their interaction with platelets and endothelial cells (45), and reduce lung colonization in

Table 1: Specific 12-LOX Interactors <sup>a</sup>							
clone	sequence	bait panel					
		12-LOX	bicoid2	tailless	hairy	cdc2	Cdi D
1	keratin K5	++++	–	–	–	–	–
2	$\beta 4$ integrin	+++	–	–	–	–	–
3	C8FW	++	–	–	–	–	–
9	$\beta 4$ integrin	+++	–	–	–	–	–
10	lamin A	+++	±	–	–	–	–
12	C8FW	++	±	–	–	–	–
15	keratin K5	++++	–	–	–	–	–
33	keratin K5	++++	–	–	–	–	–
34	keratin K5	++++	–	–	–	–	–
35	lamin A	+++	±	–	–	–	–
37	keratin K5	++++	–	–	–	–	–
38	keratin K5	++++	–	–	–	–	–

<sup>a</sup> Summary of 12-LOX interactions with the panel of baits. Interaction mating was performed as described in Materials and Methods and shown in Figure 1. Level of interaction lac Z (reporter gene activation) as determined by blue color on X-Gal indicator plates: ++++ indicates dark blue, +++ blue, ++ light blue, + very light blue, ± almost white, and – white.

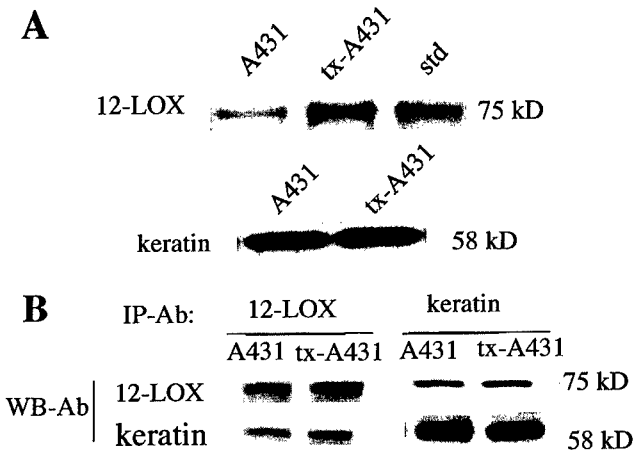


FIGURE 2: Interaction of 12-lipoxygenase and keratin in vitro. (A) Western blotting: 12-LOX (upper panel) and keratin (lower panel) protein in untransfected A431 cells (A431) and 12-LOX transfectants (tx-A431). Human recombinant platelet-type 12-LOX was used as standard (std). (B) Immunoprecipitation: 12-LOX and keratin coimmunoprecipitated from untransfected A431 cells (A431) and 12-LOX transfectant (tx-A431). Aliquots of cell lysates from either cell type were immunoprecipitated either with anti-12-LOX or anti-keratin 5 antibodies. After the samples were washed, precipitated pellets were resolved by SDS-PAGE, and proteins were detected by immunoblotting with anti-keratin 5 or anti-12-LOX antibodies. Positions of 12-LOX and keratin are indicated. The experiment was repeated three times. For each experiment, mouse, or rabbit IgG and Sepharose 4B-conjugated protein G beads alone were used as controls.

an experimental metastasis assay (46). Interestingly, treatment of tumor cells with a selective 12-LOX inhibitor (e.g., BHPP; see ref 47) also has been demonstrated to inhibit integrin expression, tumor cell platelet, and tumor cell endothelial cell interactions (48) and experimental metastasis in vivo (49). To confirm the interaction of 12-LOX with keratin in human cells, we conducted immunoprecipitation assays and confocal immunofluorescent staining in A431 cells. Meanwhile, A431 cells were transfected with a pcDNA 3.1 expression construct, containing human platelet-type 12-lipoxygenase cDNA. The overexpression of 12-LOX in A431 cells was confirmed by Western Blotting (Figure 2A). 12-LOX antibody co-immunoprecipitated keratin with 12-LOX in A431 cells and transfectants. Similarly, keratin antibody

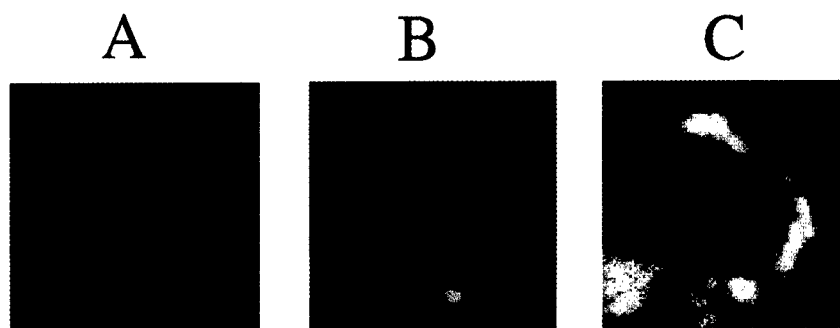


FIGURE 3: 12-LOX colocalization with keratin by laser confocal immunofluorescence images. Cells were fixed, permeabilized, and incubated first with anti-12-LOX (A) or anti-keratin (B) antibodies, then with FITC-conjugated AffiniPure goat anti-mouse IgG (A, green) or Rhodamine Red-X-conjugated AffiniPure goat anti-rabbit IgG (B, red). Merged image in C (yellow) superimposes image from the first two images. Yellow color indicates overlap between 12-LOX (green) and keratin (red).

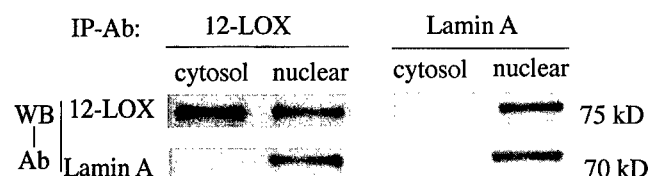


FIGURE 4: Interaction of 12-LOX and lamin A in A431 cells by immunoprecipitation assay: 12-LOX and lamin A coimmunoprecipitated from nuclear extracts of untransfected A431 cells. After the centrifugation at 25000g, the supernatants and pellets from  $5 \times 10^6$  cells were designated as either subcellular fraction of cytosol or nuclear. The supernatants and resuspended pellets were immunoprecipitated either with anti-12-LOX or anti-lamin A antibodies. After the samples were washed, precipitated pellets were resolved by SDS-PAGE and proteins were detected by immunoblotting with anti-lamin A or anti-12-LOX antibodies. Positions of 12-LOX and lamin A are indicated. The experiment was repeated three times. For each experiment, mouse or rabbit IgG and Sepharose 4B-conjugated protein G beads alone were used as controls.

co-immunoprecipitated 12-LOX with keratin in A431 cells and transfectants (Figure 2B). We also co-immunoprecipitated 12-LOX with lamin A using 12-LOX antibody and co-immunoprecipitated lamin A with 12-LOX using lamin A antibody (Figure 4). We were able to use immunofluorescent staining in A431 cells to show that 12-LOX co-localized with keratin in the cytoplasm, mostly around the cell nucleus forming a ring-like structure (in yellow), as seen in Figure 3. Combined, our results indicate that 12-LOX physically interacts with keratin in the cytoplasm and with lamin A in the nucleus of human tumor cells.

Our findings are consistent with a recent report that 12-LOX activity is detected predominantly in the particulate fractions in murine keratinocytes (50). These particulate fractions were found by ultrastructural analysis to contain mainly insoluble proteins such as keratin but not membrane structures (50). Taken together, these findings suggest that human keratin may be a novel regulator of 12-LOX activity by its effect on 12-LOX subcellular localization. The mechanism whereby keratin may affect the subcellular localization of 12-LOX is unknown. However, there are studies suggesting that intermediate filaments participate in intracellular trafficking of proteins to the plasma membrane. For example, it has been demonstrated that intermediate filaments associate and facilitate the transport of single vesicles and lipoprotein droplets in CHO, adipose and steroidogenic cells (51–52). Another study has shown that 5-LOX binding to certain cytoskeletal proteins including  $\alpha$ -actin and that actin may mediate compartmentalization and

translocation of 5-LOX in myeloid cells (53). Interestingly, we recently observed that the translocation of 12-LOX from cytosol to membrane upon stimulation in A431 cells was blocked after disrupting the keratin component of intermediate filaments (data not shown). It is tempting to speculate that keratin may be similarly involved in the transport of 12-LOX from the cytoplasm to a membrane-bound site. Hagmann et al. (41) have demonstrated 12-LOX activity in isolated nuclei, and our laboratory has demonstrated that, upon stimulation, 12-LOX associates with membrane structures and that this association results in an increase in 12-LOX activity (36, 41). We are currently attempting to further characterize the interaction between keratin and 12-LOX.

In addition to keratin, we demonstrated that 12-LOX associates with lamin A, phosphoprotein C8FW, and integrin  $\beta 4$  subunit by yeast two-hybrid screen. Like type II keratin K5, lamin A is also an intermediate filament protein and has been identified as a component of the nuclear lamin A, a meshwork of intermediate filaments on the inner surface of nuclear membranes. The association of 12-LOX with lamin A is consistent with the finding of 12-LOX activity in isolated nuclei, as mentioned above. Since keratin and lamin A both are components of cytoskeletal intermediate filaments, we hypothesize that keratin and/or lamin A may contribute to the regulated trafficking of 12-LOX. As will be described elsewhere, we also have shown by coimmunoprecipitation and confocal immunofluorescent colocalization that the  $\beta 4$  integrin interacts specifically with 12-LOX in A431 and CHO cells and that this interaction increases enzymatic activity of 12-LOX with increased 12(S)-HETE production (Tang et al., unpublished observation). When the same experiment was performed and probed with antibodies to 5-LOX or 15-LOX no association between the  $\beta 4$  integrin and 5-LOX or 15-LOX was observed (data not shown) demonstrating the specificity of 12-LOX interaction with the  $\beta 4$  integrin. The  $\beta 4$  integrin is expressed on carcinoma cells and is linked to tumor cell motility and invasion (54). Another 12-LOX interactor as determined by yeast two-hybrid analysis is the phosphoprotein C8FW. The human full-length has not been sequenced and no antibody is available. Therefore, we have not yet verified its interaction with 12-LOX in human cancer cells. A similar protein, called C5FW, has been cloned from dog thyroid cells and shares 95% amino acid identity with its human counterpart. These novel proteins can be phosphorylated after mitogenic stimulation, and meanwhile, they themselves can function as a kinase (18). It will be interesting to further characterize the

interaction between 12-LOX and these other proteins and how they may contribute to 12-LOX regulation.

In summary, we have isolated four 12-LOX interacting proteins: type II keratin K5, Lamin A, cytoplasmic domain of  $\beta 4$  integrin, and phosphoprotein C8FW. Thus far we have verified that three of these (keratin, lamin A, and  $\beta 4$  integrin) are bona fide 12-LOX binding proteins in human cancer cells. We further demonstrated 12-LOX and keratin physically interact and colocalize around the nuclear membrane. Further characterization of the individual interaction between 12-LOX and each of these four proteins will provide insights into the regulation of 12-LOX activity. Ultimately, a clearer understanding of 12-LOX regulation may permit therapeutic intervention in tumor progression and metastasis.

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## EXPRESSION, SUBCELLULAR LOCALIZATION AND PUTATIVE FUNCTION OF PLATELET-TYPE 12-LIPOXYGENASE IN HUMAN PROSTATE CANCER CELL LINES OF DIFFERENT METASTATIC POTENTIAL

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The involvement of 12-lipoxygenase (12-LOX) expression and function in tumor metastasis has been demonstrated in several murine tumor cell lines. In addition, 12-LOX expression was detected in human prostatic tumors and correlated to the clinical stage of disease. Here we provide data that human prostate cancer cell lines express the platelet-type isoform of 12-LOX at both the mRNA and protein levels, and immunohistochemistry revealed 12-LOX expression in human prostate tumors. The enzyme was localized to the plasma membrane, cytoplasmic organelles and nucleus in non-metastatic cells (PC-3 nm) and to the cytoskeleton and nucleus in metastatic cells (DU-145). After orthotopic/intra-prostatic injection of tumor cells into SCID mice, the metastatic prostate carcinoma cells (DU-145) expressed 12-LOX at a significantly higher level compared with the non-metastatic counterparts, PC-3nm. The functional involvement of 12-LOX in the metastatic process was demonstrated when DU-145 cells were pretreated *in vitro* with the 12-LOX inhibitors N-benzyl-N-hydroxy-5-phenylpentamide (BHPP) or baicalein, the use of which significantly inhibited lung colonization. These data suggest a potential involvement of 12-LOX in the progression of human prostate cancer. *Int. J. Cancer* 87: 37–43, 2000.

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Membrane receptors regulate cellular activities by triggering a cascade of events ultimately affecting the function of various kinases. Significant components of the signaling cascade are phospholipases providing arachidonate, which is further metabolized by either cyclooxygenase (COX) or lipoxygenase (LOX) enzymes. It is known that both COX (Brinkman *et al.*, 1990; Thompson *et al.*, 1984) and LOX (Honn *et al.*, 1994a; Eling and Glasgow, 1994) products are able to provide mitogenic signals to cells. Further, it has been shown that LOXs are essential regulators of cell survival and apoptosis (Tang *et al.*, 1996). However, 12-LOX and its product 12-hydroxyeicosatetraenoic acid (12-HETE) have several other physiological functions including the induction of endothelial cell retraction, platelet aggregation and chemokinesis of leukocytes (Honn *et al.*, 1994a). 12-LOX is expressed in 3 immunologically and enzymologically distinct forms (platelet, leukocyte and epithelial) which are characterized by unique tissue distributions (Takahashi *et al.*, 1998; Hamsbrough *et al.*, 1990; Hagmann, 1997).

It was shown that various rodent and human tumor types express 12-LOX, produce 12-HETE or respond to exogenous 12-HETE. These include murine melanomas (Chen *et al.*, 1994; Silletti *et al.*, 1994) and carcinomas (Chen *et al.*, 1994; Hagmann *et al.*, 1995; Wang *et al.*, 1996), human leukemias (Hagmann *et al.*, 1993), carcinomas (Chen *et al.*, 1994; Hagmann *et al.*, 1996) and melanomas (Timar *et al.*, 1999), suggesting an important function for 12-HETE in tumor biology. Systematic studies revealed that in addition to the importance of 12-HETE as a mitogenic signaling molecule, 12-LOX and 12-HETE have important roles in tumor metastasis as generators of mitogenic signals (Timar *et al.*, 1993a),

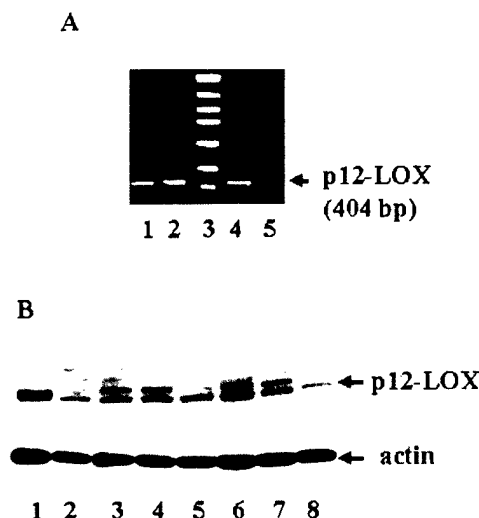


FIGURE 1—(a) RT-PCR analysis of 12-LOX mRNA in human prostate cell lines. Total RNA from PC-3 nm (lane 1), DU-145 (lane 2), HEL (as positive control; lane 4) and sample without RNA (lane 5) were PCR amplified using platelet-type-specific 12-LOX primers. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining. Lane 3 is molecular size standard (plasmid digest). (b) Western blot analysis of cellular proteins from human prostate cancer cell lines probed for platelet-type 12-LOX. Cellular proteins were isolated, separated by SDS-PAGE, transferred to nitrocellulose membrane and probed for 12-LOX protein as described in Material and Methods. Platelet lysate was used as positive control (lane 1). Control actin blot serves as protein and loading reference. ML2 (lane 2), TSU (lane 3), DU-145 (lane 4), PC-3 (lane 5), LN (lane 6) and PPC-1 (lane 7) are human prostate cancer cell lines, whereas NHP (lane 8) is a prostate epithelial cell line. Note the double bands in ML2, TSU, DU-145, LN and PPC-1 cells.

upregulation of adhesion molecules (Chopra *et al.*, 1991) and promotion of local invasion through matrices by enhancing proteolytic enzyme release (Honn *et al.*, 1994b).

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Prostate cancer has become a leading malignancy in the aging populations of the Western hemisphere, which justifies the increased interest in the biology of this disease. Previously, we demonstrated that 12-HETE promotes rodent prostate carcinoma motility and invasion (Liu *et al.*, 1994b), suggesting that this molecule might be an important component of the motogenic signaling pathway in prostate cancer, similar to melanoma (Timar *et al.*, 1993a). Therefore, the aim of our study was to establish the expression of 12-LOX in human prostate carcinoma cell lines both at the mRNA and protein levels, as well as at the protein level in human prostate tissue, and to study its subcellular localization and functional significance.

#### MATERIAL AND METHODS

##### Cell and tumor lines

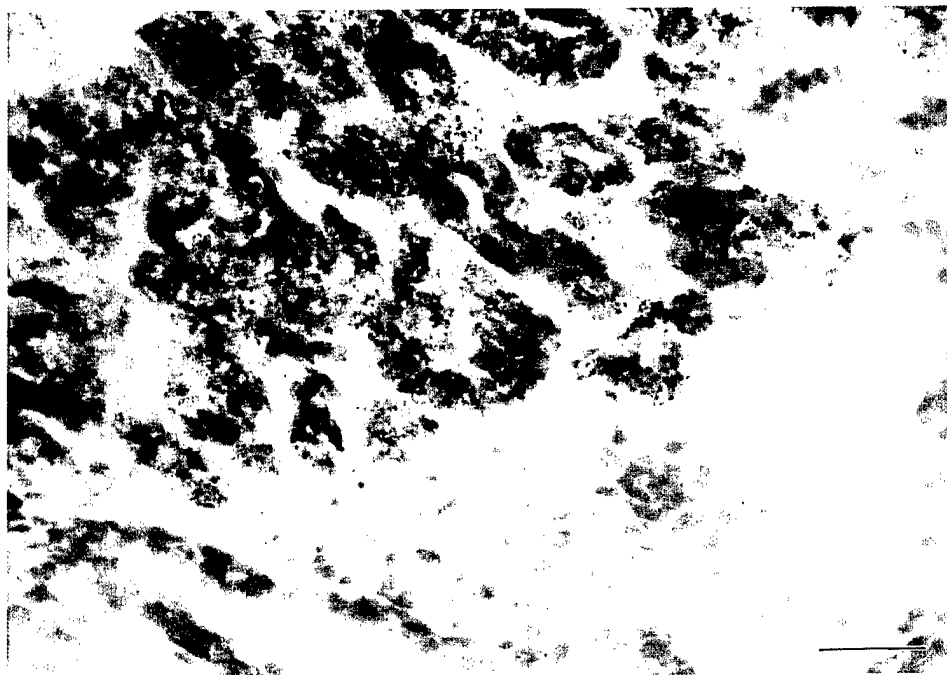
PC-3, DU-145, LNCaP and HEL cells were obtained from American Type Culture Collection (Rockville, MD) and routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics. A non-metastatic subline of PC-3 (*i.e.*, PC-3 nm) was established. The PC-3 nm line proved to be tumorigenic but non-metastatic, whereas the DU-145 cell line was both tumorigenic and metastatic *in vivo* in SCID mice after orthotopic implantation (Timar *et al.*, 1996b). Normal human prostate (NHP) epithelial cells were obtained from Clonetics (San Diego, CA) and cultured in KBM basic medium with 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract, 10 ng/ml cholera toxin and antibiotics. Primary prostate carcinoma-1 (PPC-1) cells were initially provided by Dr. A. Brothman (Eastern Virginia Medical School). The TSU cell line was provided by Dr. W.G. Nelson (Johns Hopkins Cancer Center). A PC-3 subline that preferentially metastasizes to bone (PC-3-ML2) was provided by Dr. M. Stearns (Medical College of Pennsylvania). These latter 3 cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics.

##### Detection of 12-LOX expression at RNA level

Total RNA was obtained using the guanidinium isothiocyanate-CsCl method as described previously (Hagmann *et al.*, 1996) and suspended at 1 mg/ml in distilled water. Three micrograms of RNA was reverse transcribed (RT) using oligo(dT)18 primers and 2 µl of the cDNA mixture was amplified by polymerase chain reaction (PCR) with sense and antisense primers. The primers span exon-intron boundaries of the 12-LOX gene thus avoiding amplification of genomic DNA during PCR. DNase was used to prevent PCR product carryover and genomic DNA contamination. The RT-PCR was performed in the GeneAmp PCR 9600 (Perkin-Elmer-Cetus, Wellesley, MA) at 94°C for 30 sec, 70°C for 30 sec and 72°C for 30 sec for 35 cycles. The sequence of the platelet-specific 12-LOX sense and antisense probes was described previously (Hagmann and Borgers, 1997). Fifteen microliters of PCR products was separated by electrophoresis on 2% agarose gels, stained by ethidium bromide and photographed. PCR products were sequenced directly or after cloning into the pCR2.1 TOPO II vector (Invitrogen, La Jolla, CA) and sequenced with T-7 and SP-6 primers as described (Tripathi *et al.*, 1996, 1997). The sequence was aligned to 12-LOX cDNA (Funk *et al.*, 1990) by using the MacVector 4.1 software on a Macintosh Quadra 630 computer.

##### Detection of 12-LOX expression at protein level

**Western blotting.** Adherent tumor cells cultured *in vitro* were dislodged from the flask and washed in PBS. Aliquots of the homogenate were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gels. Proteins were electrophoretically transferred to nitrocellulose membranes. Cells were lysed in TNC (10 mM Tris-acetate, 0.5% NP-40, and 5 µM CaCl<sub>2</sub>, pH 7.4) buffer on ice. The solubilized proteins were loaded on 4–20% gradient SDS-PAGE, transferred to nitrocellulose paper (the non-specific binding sites were blocked by non-fat milk) and then probed with primary antibody anti-12-LOX (Oxford Biomedical, Oxford, UK) and secondary antibody goat anti-rabbit IgG (Bio-Rad, Munich, Germany) conjugated to horseradish peroxidase. Enhanced chemiluminescence (ECL; Amersham, Little



**FIGURE 2** – Immunohistochemistry of 12-LOX performed on frozen prostate tumor. There is intense cytoplasmic positivity in the malignant glands of this Gleason score 6 adenocarcinoma. There is no immunoreactivity in the epithelium of the normal prostatic glands (N) or the stromal cells in the background. Scale bar = 50 µm.

Chalfont, UK) was used for detection. For positive controls we utilized either human erythroleukemia (HEL) cells, which have been demonstrated to express platelet-type 12-LOX (Funk *et al.*, 1990), or partially purified human platelet-type 12-LOX obtained from human platelet lysates (Oxford Biomedical).

**Flow cytometry.** Adherent cells were detached from tissue culture flasks by using low EDTA washing or isolated from solid tumors as described previously (Trikha *et al.*, 1998), centrifuged at 100g and fixed in absolute methanol for 10 min. After washing in PBS, cells were labeled for platelet-type 12-LOX using a rabbit polyclonal antibody, biotinylated goat anti-rabbit IgG (Amersham) and Streptavidin-FITC (Amersham). For negative controls, the primary antibody was omitted. Flow cytometry of the samples was performed as described previously (Timar *et al.*, 1993a), wherein 90% of the fluorescence of the negative control cell population

served as the gate. Both percent of positive cells and fluorescence intensity were determined. Samples were run in triplicate.

**Confocal laser scanning microscopy.** Tumor cells were plated onto fibronectin-coated glass coverslips *in vitro* for 60 min at 37°C in serum-free RPMI medium, washed and the adherent cells were fixed in 1% paraformaldehyde (PFA)/PBS or absolute methanol for 10 min. The PFA-fixed cells were permeabilized by 0.1% Triton X-100/PBS (4 min). After washing in PBS, coverslips were blocked with 10% non-immune goat serum/PBS for 30 min at room temperature. Platelet-type 12-LOX was identified by immunocytochemistry using the polyclonal anti-12-LOX antibody as above. The primary antibody was diluted 1:100 in 3% bovine serum albumin (BSA)/PBS and the cells were incubated for 60 min at room temperature. After repeated washings in PBS, the bound antibody was detected using a biotinylated goat anti-rabbit IgG (Amersham) and Streptavidin-FITC (Amersham). Control cells were incubated with non-immune rabbit serum alone. To promote the subcellular localization of the enzyme, cells were counterstained with propidium iodide to identify the DNA-containing nucleus. Confocal microscopy was performed on a Bio-Rad MRX 1024 confocal microscope operating LaserSharp software (Bio-Rad).

#### Detection of 12-LOX expression in human prostate cancer

**Immunohistochemistry.** Sections were cut from snap-frozen human prostate cancer tissue at 4  $\mu$ m thickness and placed on gelatin-coated glass slides. Prostate tissues from radical prostatectomy specimens removed from a total of 18 patients were examined. The sections were fixed in 100% cold acetone for 10 min. Primary antibody against 12-LOX (Oxford Biomedical) was diluted  $\times 100$  and incubated for 20 min. Detection was performed with an ABC kit (Vector Labs, Burlingame, CA).

#### Inhibition of 12-LOX in vitro

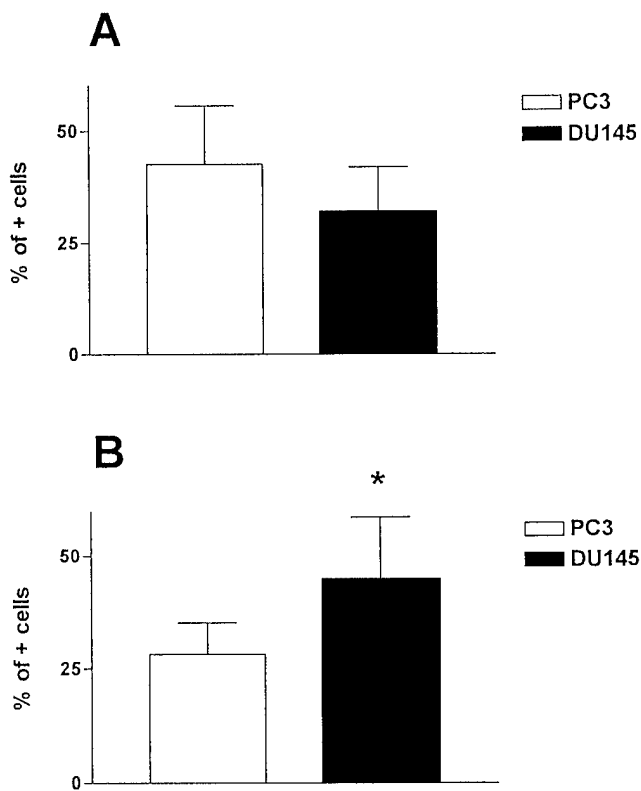
The select 12-LOX inhibitors used were N-benzyl-N-hydroxy-5-phenylpentanamide (BHPP; Biomide Corp., Grosse Pointe Farms, MI; Chen *et al.*, 1994; Liu *et al.*, 1994) and baicalein (BioMol, Plymouth Meeting, PA; Kimura *et al.*, 1987). Cultured tumor cells were treated with the 12-LOX inhibitors BHPP (Chen *et al.*, 1994), or baicalein (Kimura *et al.*, 1987) dissolved in EtOH for 60 min at 37°C in serum-free RPMI at a concentration of 5  $\mu$ M. This concentration was derived from previous studies (Chen *et al.*, 1994). Control cultures were treated with ethanol solvent alone.

#### Orthotopic injection, re-isolation of tumor cells and flow cytometry

Cultured tumor cells were detached from the plastic surface by low EDTA (Timar *et al.*, 1993a), resuspended in RPMI and  $5 \times 10^5$  tumor cells injected into the prostate of SCID mice in 50  $\mu$ l volume under anesthesia by Nembutal. On the 8th week of the experiment animals were sacrificed by Nembutal overdose, the 5–10-mm-sized primary tumors were surgically removed, sliced with crossed scalpels in RPMI and digested with collagenase type IV (Sigma, St. Louis, MO), testicular hyaluronidase (Sigma) and DNase (Sigma). A single cell suspension was prepared by repeated centrifugations and the contamination of the human tumor suspension with host mouse cells was determined by flow cytometric measurement of murine transplantation antigen H2d-expressing cells. These studies indicated that the host cell contamination was always less than 15%.

#### Lung colonization assay

*In vitro* cultured tumor cells were detached from culture dishes by using low EDTA, washed in RPMI and injected into the tail vein of SCID mice at a dose of  $10^5$  tumor cells/mouse. Animals were sacrificed by Nembutal overdose on the 30th day and the internal organs were analyzed for  $10^5$  tumor colonies. Histologic analysis was performed on the lung tissue to confirm the presence of prostate carcinoma metastases. In view of the huge tumor mass



**FIGURE 3** – Flow cytometric analysis of 12-LOX expression at protein level in human prostate cancer cell lines. (a) *In vitro* cultured cells. *In vitro* cultured tumor cells were detached from the plastic surface, suspended, fixed in methanol and labeled for platelet-type 12-LOX using a polyclonal anti-human 12-LOX-specific rabbit antibody. The bound antibody was revealed by a biotinylated secondary antibody and FITC-labeled Streptavidin. Cells ( $10^4$ ) were analyzed by flow cytometry from each sample for the percent of positive cells. Gate was set according to the fluorescence intensity of the negative controls. Data are expressed as means  $\pm$  SD ( $n = 3$ ). (b) *In vivo* growing primary tumors. Tumor cells were injected orthotopically into the prostate of SCID mice, 5–10-mm-sized primary tumors were removed and tumor cells were isolated by an enzymatic method (see Material and Methods). Single cell suspensions were fixed in methanol and labeled for platelet-type 12-LOX using a polyclonal anti-human 12-LOX-specific rabbit antibody. The bound antibody was revealed by a biotinylated secondary antibody and FITC-labeled Streptavidin. Cells ( $10^4$ ) were analyzed by flow cytometry from each sample for the percent of positive cells. The gate was set according to the fluorescence intensity of the negative controls. Platelets and red blood cells were gated out from the measurement by their light scatter characteristics. Data are expressed as means  $\pm$  SD ( $n = 3$ ). \* $p < 0.05$ .

TABLE 1—INVASIVE PHENOTYPE OF HUMAN PROSTATE CANCER CELL LINES PC-3 AND DU-145 IN THE PROSTATE OF SCID MICE<sup>1</sup>

Type of invasion	PC-3 nm	DU-145 (%)
Prostate capsule	Not detected	100
Rectum	Not detected	50
Muscle	Not detected	60
Vascular	Not detected	40

<sup>1</sup>Data represent the incidence of local invasion.

in the lungs, the weight of the organ was determined in all experimental groups.

## RESULTS

The expression of 12-LOX mRNA was examined in HEL cells, low metastatic PC-3 cells (*i.e.*, PC-3 nm) and high metastatic prostate cancer cells (DU-145). Total RNA was isolated from each cell line, reversed transcribed to cDNA and amplified by PCR using platelet-type 12-LOX-specific primers, as described in Material and Methods. The RNA from HEL, PC-3 nm and DU-145 cells all yielded the predicted 404 bp size fragment (Fig. 1a). The authenticity of this fragment was confirmed by Southern blot analysis with a human 12-LOX cDNA probe (data not shown). The fragment shared 100% homology with human platelet-type 12-LOX (Funk *et al.*, 1990) within the sequenced region (data not shown).

The presence of platelet-type 12-LOX in prostate carcinoma cells was further confirmed by Western blotting of cellular proteins using 6 *in vitro* cultured human prostatic carcinoma cell lines. The apparent size of the band (73 kDa) corresponded to partially purified human platelet-type 12-LOX isolated from platelet lysates (Fig. 1b). A positive band was detected in ML-2, TSU, DU-145, PC-3 (wild type), LN and PPC-1 prostate carcinoma cells. A minor amount of 12-LOX was also detected in NHP epithelial cells (Fig. 1b). An upper band of approximately 75 kDa was detected in TSU, DU-145, LN and PPC-1 cells. This may represent a phosphorylated form of platelet-type 12-LOX (K. Tang and K.V. Honn, unpublished observation).

The above data demonstrate that authentic platelet-type 12-LOX is expressed at both the message and protein levels in *in vitro* cultured prostate carcinoma cells. To confirm that the expression of platelet-type 12-LOX was not an artifact of *in vitro* cell culture, we performed immunohistochemical analysis of radical prostatectomy specimens. A total of 12 of 18 patient tumors stained positive for 12-LOX. A representative section is shown in Figure 2. Positive staining was observed in the tumor cells with little or no staining in normal tissue.

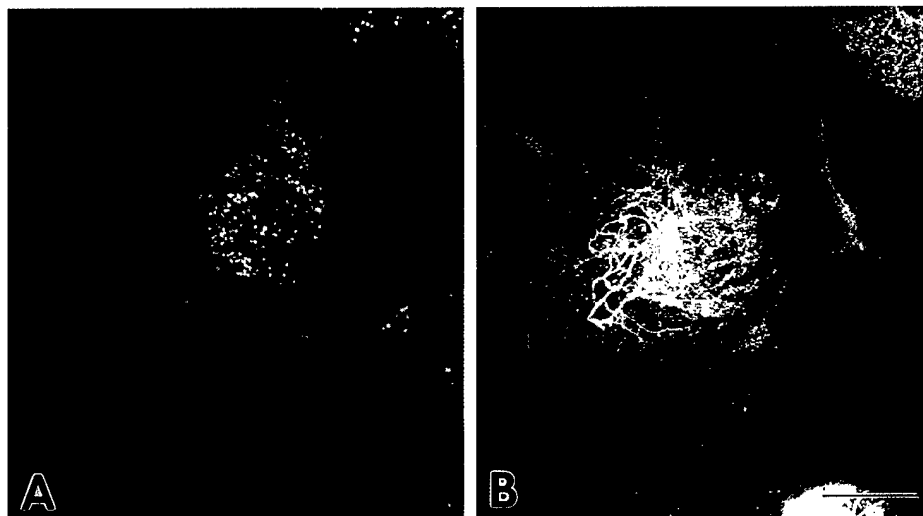
After we established the presence of platelet-type 12-LOX both *in vitro* and *in vivo* in prostate cancer, further studies in this report focused on the PC-3 nm and DU-145 cell lines. Immunocytochemistry and flow cytometry indicated that 30–40% of cultured tumor cells express the platelet-type 12-LOX (Fig. 3a). Interestingly, when tumor cells were injected into the prostate of SCID mice and the tumor cells re-isolated from the primary tumor, flow cytometry detected 12-LOX expression in DU-145 cells at a significantly higher proportion compared with the PC-3 nm cell line (Fig. 3b), suggesting that the local environment may differentially regulate the expression of the enzyme at the protein level in these 2 cell lines. We next compared the subcellular localization of 12-LOX in PC-3 nm and DU-145 cells. Confocal microscopy revealed that the 12-LOX protein in PC-3 nm cells can be detected at the peripheral plasma membrane of spread tumor cells, at vesicular cytoplasmic organelles and in the interchromatic space of the nucleus (Fig. 4a). In DU-145 cells, the 12-LOX protein was also observed at the peripheral plasma membrane and in the nucleus; however, the protein was found predominantly in the cytoplasm forming filaments which appeared to surround the nucleus (Fig. 4b).

*In vivo* PC-3 nm cells are tumorigenic but not invasive, whereas DU-145 cells are both tumorigenic and metastatic (Timár *et al.*, 1996). *In vivo* studies indicated that the 2 tumor cell lines have different invasive potential after intraprostatic injection (Table I). PC-3 nm tumor did not penetrate the prostatic capsule and never progressed beyond that border (Fig. 5a). In contrast, DU-145 tumors always infiltrated the prostatic capsule and in 50% of the cases it was possible to find evidence of local invasion in the muscle (Fig. 5b), adipose tissues or rectum (data not shown).

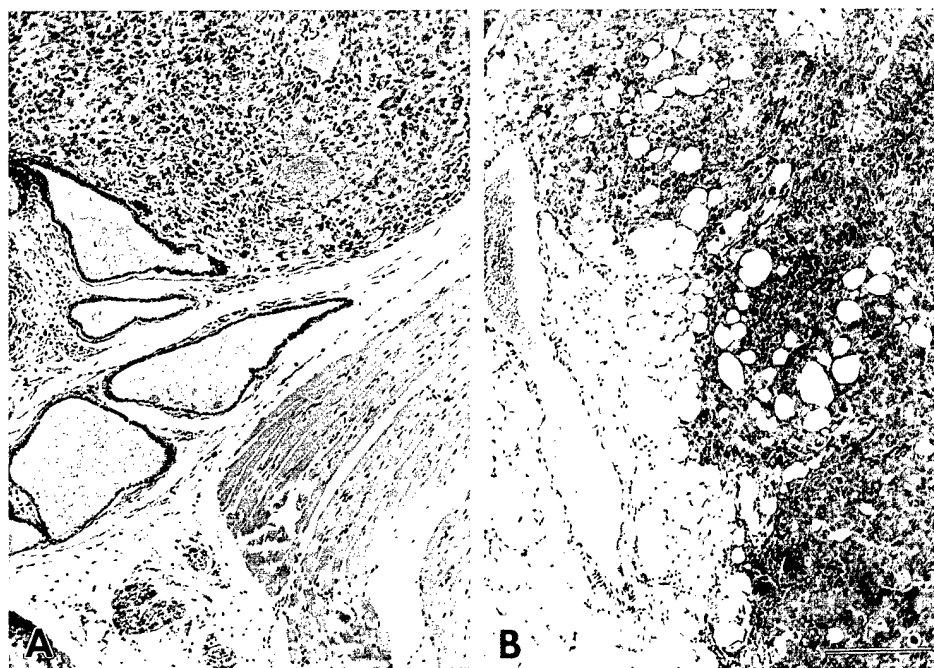
Since we observed that after intraprostatic growth the metastatic DU-145 cells express 12-LOX protein at a significantly higher level compared with the PC-3 nm cells (Fig. 3b), we postulated that this may have functional significance. Therefore, *in vitro* cultured DU-145 tumor cells were pretreated with the LOX inhibitors baicalein or BHPP *in vitro* (60 min; 5  $\mu$ M) and tested for their lung colonization potential. Measurement of the tumor-bearing lungs 30 days after injection indicated that both agents reduced lung colonization and the effect of BHPP proved to be statistically significant (Fig. 6).

## DISCUSSION

Expression of platelet-type 12-LOX in the human prostate cancer cell line PC-3 was demonstrated recently (Nie *et al.*, 1998). In that study, high expressor clones were produced by stable transfection with platelet-type 12-LOX. These high expressor clones exhibited an increased *in vivo* growth due to enhanced tumor-induced angiogenesis (Nie *et al.*, 1998). In this study, we provided evidence for the presence of 12-LOX message in 2 cell lines (PC-3 and DU-145), and 12-LOX protein in 6 prostate cancer cell lines. We compared the expression of 12-LOX in a metastatic human prostate cancer cell line, DU-145, with a non-metastatic counterpart, PC-3 nm, a subline of PC-3, and have provided evidence that DU-145 cells, similar to PC-3 nm, not only express the platelet-type 12-LOX gene but also contain the enzyme protein. RT-PCR indicated the presence of the same message in prostate cancer cell lines PC-3 nm and DU-145 as in the positive control HEL cells. Furthermore, Western blotting revealed a predominant protein band in both tumor lines using an anti-12-LOX antibody. However, the molecular weight of the predominant band in both prostate cancer cell lines was lower (73 kDa) than the one in HEL cells (75 kDa). Lower molecular weight 12-LOX protein bands in tumor cell lines were observed previously in 3LL murine carcinoma cells (Hagmann *et al.*, 1995). Furthermore, a 12-LOX of 68 kDa size was isolated recently from rat skin cells (Lomnitski *et al.*, 1995). Explanation for the lower molecular mass of these proteins could be various phosphorylation states, presence of splice variants or partial protein degradation. The significance of our finding of 12-LOX expression in human prostate cancer cells can be fundamental from both a prognostic and a therapeutic point of view. Endogenous production of 12-HETE was correlated to the invasive and metastatic potential of murine tumors (Chen *et al.*, 1994; Hagmann *et al.*, 1993). Further, it was also demonstrated that the enzyme which initiates 12-HETE production is actually 12-LOX (Chen *et al.*, 1994; Hagmann *et al.*, 1993, 1996). On the other hand, 12-LOX expression in tumors is not restricted to rodents, since various human tumor cell lines have been found to express 12-LOX (mostly the platelet-type isoform) including epidermoid carcinoma (Hagmann *et al.*, 1996), colorectal adenocarcinoma (Chen *et al.*, 1994), melanomas (Timár *et al.*, 1999) and erythroleukemias (Hagmann *et al.*, 1993). Transfection of platelet-type 12-LOX into PC-3 nm cells increased tumor-induced angiogenesis (Nie *et al.*, 1998), whereas *in vivo* treatment of animals with a select 12-LOX inhibitor resulted in a delayed growth of DU-145 cells in the prostate (Tang *et al.*, 1998) suggesting a role for platelet-type 12-LOX in the regulation of growth of prostate cancer. On the other hand, in contrast to several rodent tumor types, there are no data on non-hemopoietic human tumors concerning a possible function of 12-LOX in tumor metastasis. Therefore, it is



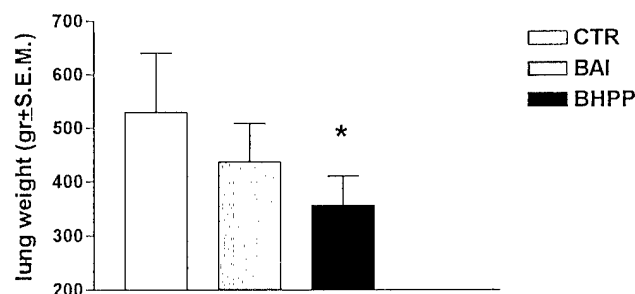
**FIGURE 4** – Localization of 12-LOX in cultured prostate cancer cells by confocal laser scanning microscopy. (a) Informative optical section of a fibronectin-adherent PC-3 nm cell. 12-LOX protein (green fluorescence) appears in the nucleus (red fluorescence) in the interchromatic spaces, in the cytoplasm in the form of heterogeneous vesicles and at the cell membrane. (b) Informative optical section of a fibronectin-adherent DU-145 cell. 12-LOX protein (green fluorescence) appears in the nucleus (red fluorescence) and in the cytoplasm as filaments or individual granules. Note the accumulation at the cell center (arrow). Fibronectin-adherent cells were fixed in MetOH, blocked for non-specific binding and labeled with a polyclonal rabbit IgG produced against platelet-type 12-LOX diluted 1:100; the bound antibody was detected by a biotinylated anti-rabbit IgG and FITC-conjugated Streptavidin (dilution 1:100). Nuclei were detected by propidium iodide post-staining. Confocal images were obtained by using the merged red and green signals superimposed on the phase contrast image. Scale bar = 10  $\mu$ m.



**FIGURE 5** – Histology of the intraprostatic human prostate cancer xenografts. (a) PC-3 nm tumor. Peripheral area of the tumor in the SCID mouse prostate 4 weeks after intraprostatic injection. Note the intact fibrous capsule between the tumor (lower left area), the prostate gland (upper left area) and the muscular tissue (right). (b) DU-145 tumor. Peripheral area of the tumor in the SCID mouse 4 weeks after intraprostatic injection. Note the anaplastic tumor cell nests infiltrating the periprostatic muscular tissue. Hematoxylin-eosin stains. Scale bar = 25  $\mu$ m.

important that we have shown in this report that a significant proportion of the human prostate cancer cells expresses 12-LOX. Furthermore, the metastatic cell line DU-145, implanted into the prostate of SCID mice, expressed 12-LOX at a significantly higher level than the non-metastatic counterpart, PC-3nm, suggesting that

the enzyme might be functional in the metastatic cascade similar to what was observed previously in several rodent tumors (Honn *et al.*, 1994a). This assumption was further corroborated in *in vivo* experiments where we have shown that *in vitro* pretreatment of human prostate carcinoma cells with 12-LOX inhibitors dimin-



**FIGURE 6**—Effect of LOX inhibitor pretreatments of DU-145 cells on lung colonization in SCID mice. Tumor cells were pretreated *in vitro* for 60 min with 5  $\mu$ M baicalein (BAI), 5 mM BHPP or solvent (CTR). Pretreated tumor cells were injected into the tail vein of SCID mice at  $10^6$  cells/animal. The experiment was terminated on the 30th day, and lungs were removed. Due to the large amount of tumor nodules in the lungs of the control animals, the weight of the lungs was determined to quantitate the lung colonization. Data are expressed in grams and are means  $\pm$  SD of 8–10 animals. \* $p < 0.05$ .

ished their lung colonization potential. The most effective inhibitor, BHPP, was shown previously to be antimetastatic in the case of murine melanoma at comparable *in vitro* dose ranges (*i.e.*, 5–10  $\mu$ M) (Chen *et al.*, 1994).

This study also provided novel data on the subcellular distribution of 12-LOX in tumor cells. Previously, 12-LOX was localized to the apical plasma membrane, to the cytosol and to the nucleus (Hagmann, 1997; Hagmann *et al.*, 1996). Here we have demonstrated that in matrix adherent prostate carcinoma cell lines 12-LOX is also present in the plasma membrane, at cytoplasmic vesicles and at cytoskeletal filaments. The presence at the plasma membrane can be correlated to the known signaling function of 12-HETE and 12-LOX (Liu *et al.*, 1994a), while the presence at the nucleus may be correlated to its role in cell survival and apoptosis (Tang *et al.*, 1996; Hagmann, 1997). The novel finding that 12-LOX is also associated to cytoplasmic vesicles and cytoskeletal filaments might explain those observations that 12-HETE has regulatory effects on organelle translocation or cytoskeletal rearrangements (Timar *et al.*, 1993b). It is noteworthy to mention in this respect that biochemical studies suggested that almost all biological effects of 12-HETE are mediated through protein kinase C( $\alpha$ ) (Liu *et al.*, 1995), which was also found to be associated not only to the plasma membrane but to the cytoskeleton and cytoplasmic vesicles (Timar *et al.*, 1996a). Collectively, these observations suggest that a 12-LOX/protein kinase C complex might serve as a translocating signal unit acting not only at the plasma membrane but at receptor-containing vesicles, the cytoskeleton or in the nucleus. Furthermore, the system may have important functions in the progression of human prostate cancer.

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